Universidad Autónoma de Madrid
Programa de Doctorado en Biociencias Moleculares

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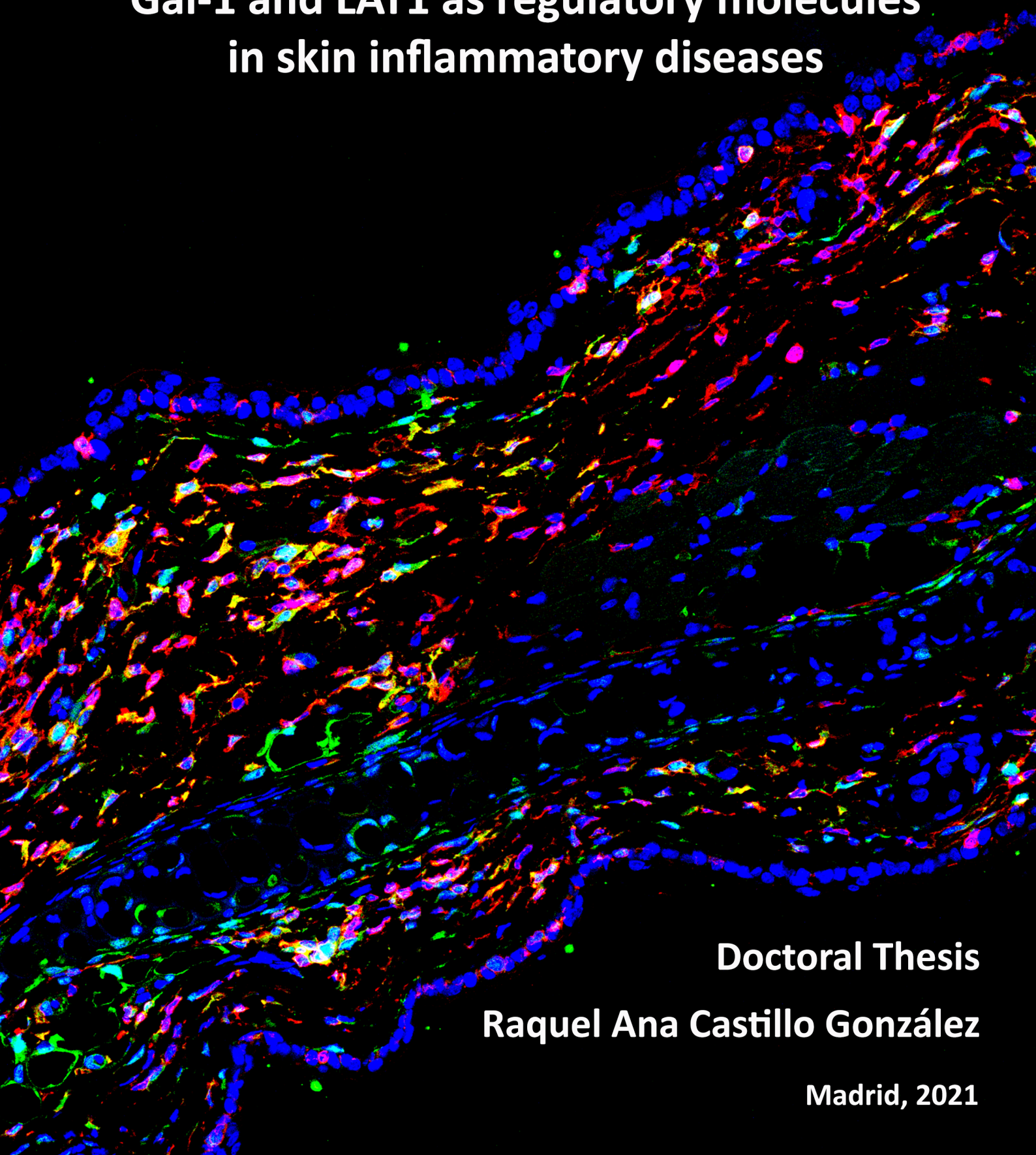
Gal-1 and LAT1 as regulatory molecules in skin inflammatory diseases



Doctoral Thesis

Raquel Ana Castillo González

Madrid, 2021

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Memoria presentada por la graduada en Biología:

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A mis padres, hermanos y sobrinos

A mi abuelo Joaquín

A Rodrigo

«Cuida tus pensamientos, porque se convertirán en tus palabras. Cuida tus palabras, porque se convertirán en tus actos. Cuida tus actos, porque se convertirán en tus hábitos. Cuida tus hábitos, porque se convertirán en tu destino»

Mahatma Gandhi

«Mira profundamente en la naturaleza y entonces comprenderás todo mejor»

Albert Einstein

AGRADECIMIENTOS

AGRADECIMIENTOS

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SUMMARY

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Skin diseases are common disorders with a high prevalence among occupational diseases. Allergic contact dermatitis (ACD) and psoriasis are complex and frequent inflammatory skin pathologies in which the immune system and epithelial alterations play an important role in their development. ACD, also known as contact hypersensitivity (CHS), is a frequent T-cell mediated inflammatory skin disease characterized by red, itchy, swollen and cracked skin. It is caused by the direct contact with an allergen and/or irritant. Galectin-1 (Gal-1) is a β-galactoside-binding lectin which is highly expressed in several types of immune cells. The role of endogenous Gal-1 in CHS is not known. We found that Gal-1-deficient mice display more sustained and prolonged skin inflammation than wild-type mice after oxazolone treatment. Gal-1-deficient mice have increased $CD8^+$ T cells and neutrophilic infiltration in the skin. After the sensitization phase, Gal-1-depleted mice showed increased frequency of central memory $CD8^+$ T cells and IFNγ secretion by $CD8^+$ T cells. The absence of Gal-1 does not affect migration of transferred $CD4^+$ and $CD8^+$ T cells from the blood to the lymph nodes or to the skin. Depletion of $CD4^+$ T lymphocytes as well as adoptive transfer experiments demonstrated that endogenous expression of Gal-1 on $CD8^+$ T lymphocytes exerts a major role in the control of CHS model. These data underscore the protective role of endogenous Gal-1 in $CD8^+$ but not $CD4^+$ T cells in the development of CHS disease.

Psoriasis is a common chronic skin disorder characterized by a thickened epidermis caused by keratinocyte (KC) proliferation and dermal inflammatory infiltrate. The main clinical manifestation is the presence of raised, squamous, erythematous oval plaques. The progress of this pathology can be affected by diverse causes such as immune system, genetic, autoantigens and environmental factors. It is mainly mediated by IL-23, IL-1β, and IL-17. Although psoriasis is a hyperproliferative skin disorder, the possible role of amino acid transporters has remained unexplored. Thus, we sought to investigate the role of the essential amino acid transporter L-type amino acid transporter 1 (LAT1) in psoriasis. We found that LAT1 expression is increased in KCs and skin-infiltrating lymphocytes of psoriatic lesions in human subjects and mice. LAT1 deletion in KCs does not dampen the inflammatory response or their proliferation, which could be maintained by increased expression of the alternative amino acid transporters LAT2 and LAT3. Specific deletion of LAT1 in γδ and $CD4^+$ T cells controls the inflammatory response induced by imiquimod (IMQ). LAT1 deletion or inhibition by JPH203 blocks expansion of IL-17-secreting $γ4^+δ4^+$ and $CD4^+$ T cells and dampens the release of IL-1β, IL-17, and IL-22 in the IMQ-induced model. Moreover, inhibition of LAT1 blocks expansion of human γδ T cells and IL-17 secretion by human $CD4^+$ T cells. IL-23 and IL-1β stimulation upregulates LAT1 expression and induces mTOR activation in $IL\text{-}17^+$ γδ and Th17 cells. Deletion or inhibition of LAT1 efficiently controls IL-23- and IL-1β-induced phosphatidylinositol 3-kinase/AKT/mTOR activation independent of T-cell receptor signaling.

Overall, this thesis work underscores the protective role of endogenous Gal-1 in $CD8^+$ T cells in the development of CHS and the novel strategy to control skin inflammation in psoriasis mediated by the IL-23/IL-1β/IL-17 axis through targeting LAT1-mediated amino acid uptake.

RESUMEN

RESUMEN

Las enfermedades de la piel son trastornos comunes y con una alta prevalencia entre las enfermedades profesionales. La dermatitis de contacto alérgica (ACD, en inglés) y la psoriasis son patologías inflamatorias complejas en las que el sistema inmunitario y las alteraciones epiteliales juegan un papel importante en su desarrollo. La ACD, también llamada hipersensibilidad por contacto (CHS, en inglés), está mediada por las células T y caracterizada por la aparición de piel rojiza, hinchada y agrietada. Está causada por el contacto directo con un alérgeno y/o irritante. La galectina-1 (Gal-1) es una lectina que se une a β-galactósidos y que se expresa en diferentes células del sistema inmunitario. Sin embargo, se desconoce el papel de Gal-1 endógena en el desarrollo de CHS. En nuestros estudios se observó que los ratones deficientes en Gal-1 mostraban una inflamación más prolongada que los ratones silvestres tras el tratamiento con oxazolona. Los ratones deficientes en Gal-1 también presentaban mayor infiltración de neutrófilos y linfocitos $CD8^+$ en la piel. Tras la fase de sensibilización, los ratones deficientes en Gal-1 mostraron un incremento en la frecuencia de linfocitos de memoria central $CD8^+$ y de secreción de IFNγ por parte de los linfocitos $CD8^+$ efectores. Sin embargo, la ausencia de Gal-1 no afectó a la migración de las células T transferidas desde la sangre a los nódulos linfáticos o a la piel. Los experimentos de depleción de células $CD4^+$ y los de transferencia adoptiva demostraron que la expresión endógena de Gal-1 en linfocitos $CD8^+$ ejerce un papel importante en el control del modelo de CHS. Por tanto, estos resultados demuestran el papel protector de Gal-1 endógena en los linfocitos T $CD8^+$, pero no en los linfocitos T $CD4^+$, en el desarrollo de la CHS.

La psoriasis es una enfermedad crónica caracterizada por el engrosamiento de la epidermis debido a la proliferación de los queratinocitos (KCs, en inglés) y al infiltrado inflamatorio de la dermis. Se manifiesta con placas ovales, elevadas, eritematosas y escamosas. Su progreso puede estar afectado por el sistema inmunitario, autoantígenos y factores genéticos y ambientales. Es un trastorno hiperproliferativo de la piel mediado por citoquinas como IL-23, IL-1β e IL-17, pero se desconoce el papel de los transportadores de aminoácidos. Por tanto, investigamos el papel del transportador de aminoácidos de tipo L 1 (LAT1) en la psoriasis. La expresión de LAT1 está incrementada en KCs y linfocitos T en lesiones psoriásicas humanas. La deleción de LAT1 en los KCs no afecta a su proliferación ni a la respuesta inflamatoria, pudiendo estar compensada esta deficiencia de LAT1 por el incremento de otros transportadores como LAT2 y LAT3. La deleción de LAT1 en células T $CD4^+$ y γδ controla la respuesta inflamatoria en el modelo de psoriasis inducido por imiquimod (IMQ). Tanto la deleción como la inhibición farmacológica (JPH203) de LAT1 bloquean la expansión de células T IL-17^+ $\gamma4^+\delta4^+$ y $CD4^+$ y, disminuye la secreción de IL-1β, IL-17 e IL-22 en el modelo de IMQ. Además, JPH203 bloquea la expansión de células T γδ humanas y la secreción de IL-17 por las células T $CD4^+$. La estimulación mediada por IL-23 e IL-1β aumenta la expresión de LAT1 y la activación de mTOR en células IL-17^+ T γδ y Th17. La deleción o inhibición de LAT1 controlan la activación del eje fosfatidilinositol 3-quinasa/AKT/mTOR inducido por IL-23 e IL-1β de manera eficaz e independiente de la señalización a través del receptor de células T.

En resumen, los resultados obtenidos desvelan el papel protector de la expresión de Gal-1 en las células T $CD8^+$ efectoras en CHS y, una nueva estrategia para controlar la inflamación de la piel en psoriasis mediada por el eje IL-23/IL-1β/IL-17 a través de la inhibición de la entrada de aminoácidos mediada por LAT1.

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LIST OF ABBREVIATIONS

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AA: amino acid

AAAs: anionic AAs

ACD: allergic contact dermatitis

ACTB: β-actin

AD: atopic dermatitis

AHR: aryl hydrocarbon receptor

AKT: protein kinase B

AMPS: antimicrobial peptides

APC: antigen presenting cell

Arg: arginine

Asp: aspartic acid

ATP: adenosine triphosphate

BrdU: bromodeoxyuridine

CAAs: cationic AAs

CAMP: cathelicidin antimicrobial peptide

CATs: cationic amino acid transporters

CCL20: CC-chemokine ligand 20

CD: contact dermatitis

CHS: contact hypersensitivity

CRD: carbohydrate-recognition domain

CXCL8: C-X-C Motif Chemokine Ligand 8

DC(s): dendritic cell(s)

dDCs: dermal dendritic cells

DETC: dendritic epidermal T cell

dLN(s): draining lymph node(s)

DMSO: dimethyl sulfoxide

DNFB: 2,4-dinitro-1-fluorobenzene

EAA: essential AA

FITC: fluorescein isothyocyanate

fl: floxed alele

FOS: fructo-oligosaccharide

Gal-1: Galectin-1

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

GCN2: general control nonderepressible 2

Gln: glutamine

Glu: glutamic acid

GM-CSF: granulocyte-macrophage colony-stimulating factor

H&E: hematoxylin and eosin

HATs: heterodimeric amino acid transporters

HCR: α-helical coiled-coil rod

His: histidine

HLA-C: human leukocyte antigen-C

ICAM-1: intercellular adhesion molecule 1

ICD: irritant contact dermatitis

IFNγ: interferon-γ

IL-: interleukin-

IL-1R: IL-1 receptor

IL-17R: IL-17 receptor

IL-23R: IL-23 receptor

IMQ: Imiquimod

Ile: isoleucine

i.p.: intraperitoneal

i.v.: intravenous

JAMs: junctional adhesion molecules

K5: Keratin 5

KCs: keratinocytes

LATs: L-type / large neutral amino acid transporters

LAT1: large neutral amino acid transporter 1

LCs: langerhans cells

Leu: leucine

LNAAs: large neutral AAs

LPS: lipopolysaccharide

Lys: lysine

L-Leu: L-leucine

L-Trp: L-tryptophan

MAPK: mitogen-activated protein kinase

mDCs: myeloid DCs

MDDCs: monocyte-derived DCs

Met: methionine

MHC: major histocompatibility complex

mTOR: mammalian target of rapamycin

NEAA: non-essential AA

NF-κB: nuclear factor κ-light-chain-enhancer of activated B cells

NKs: natural killer cells

NKT: natural killer T cell

NLRs: nucleotide-binding oligomerization domain (NOD)-like receptors

OVA: ovalbumin

OXZ: oxazolone

pDCs: plasmacytoid dendritic cells

Phe: phenylalanine

PI3K: phosphatidylinositol 3-kinase

PMA: Phorbol 12-myristate 13-acetate

PMNs: polymorphonuclear leukocytes

Pro: proline

PRR: pattern recognition receptor

P-S6: phospho-S6 ribosomal protein

PUVA: psoralen plus ultraviolet A

RANK: receptor activator of –NF-κB

RANKL: receptor activator of –NF-κB ligand

Rapa: rapamycin

rGal-1: recombinant Gal-1

RORγt: retinoic acid receptor-related orphan receptor gamma t

ROS: reactive oxygen species

SCID: Severe Combined Immune Deficiency

SD: Standard deviation

SEM: Standard error of mean

SLC: solute carrier

SNAAs: small neutral AAs

TCR: T-cell receptor

TCRαβ / αβ T cells: TCR alpha beta cells

TCRγδ / γδ T cells: TCR gamma delta cells

TGF-β: transforming growth factor beta

Th: T helper

Thr: threonine

TLR(s): Toll-like receptor(s)

TMD: transmembrane domain

TNCB: 2,4,6-trinitrochlorobenzene

TNF: tumor necrosis factor

Tom: tdTomato

Tr1: type 1 regulatory Th cells

Treg: regulatory T cell

TRM: resident memory T cells

Trp: tryptophan

Tyr: tyrosine

UVB: ultraviolet B

Val: valine

WT: wild-type

ZO-1: zonula occludens-1

γδIFNγ: IFNγ^+ γδ T cells

γδ17: IL-17$^+$ γδ T cells

INTRODUCTION

1. INTRODUCTION

1.1. SKIN AS AN IMMUNE ORGAN

The skin is a large organ of the human body, accounting for approximately 15 % of total body weight. Skin is a barrier between the body and the external environment. In addition, it participates not only in metabolic and immunological processes, but also in permeability and thermoregulatory functions (1-3). In order to accomplish its protective function against the entrance of exogenous substances and physical factors, skin makes use of physical barriers, biomolecules, a weakly acidic pH, and a network of resident immune and non-immune cells and skin structures (2).

Skin structure. To exert an effective defense against external agents, the skin shows a complex structure divided into three layers (2, 4):

- **Epidermis**. It is the peripheral layer and defends the organism from the environment. This layer is divided into five sublayers that participate in the continuous reconstruction of the skin surface: *stratum basale, stratum spinosum, stratum granulosum, stratum lucidum* and *stratum corneum*. The main cell type in these layers is the keratinocyte (KC), representing about 80 % of cells in the epidermis (2, 3, 5, 6).
    - ***Stratum basale.*** It is the deepest layer of the epidermis that contains column-shaped KCs. Basal cells are defined by their dark-staining oval or elongated nuclei and the presence of melanin pigment transferred from melanocytes (dendritic, pigment-synthesizing cells). Besides, this basal layer presents mitotically active stem cells, which are continuously generating KCs to replace those in the *stratum corneum*. Moreover, this layer shows Merkel cells (oval-shaped type I mechanoreceptors), immune cells such as T lymphocytes, and Langerhans cells (LCs). LCs are dendritic cells (DCs), which do not form cellular junction with adjacent cells. They constitute 2-8 % of the total epidermal cell population and they are the first line of defense because they can recognize and process antigens found in the epidermis. The *stratum basale* is separated from the dermis by the basal lamina and attached to it by hemidesmosomes.
    - ***Stratum spinosum.*** It is characterized by 5-10 cell layers. This epidermal layer is constituted by many cells that present different shapes, structures and subcellular features determined by their localization. In the basal part, cells are irregular and polyhedral with a rounded nucleus. In the upper spinous layer, cells are larger with lamellar granules, which are organelles with glycoproteins, glycolipids and hydrolytic enzymes. In addition, this layer presents DCs, which can capture antigens located in the damaged skin and alert the immune system to their existence.
    - ***Stratum granulosum.*** It is the most superficial layer of the epidermis with alive cells and contains 1-3 layers occupied by granular diamond-shaped cells. Cells of this layer have keratohyalin granules with keratin precursors and lamellar granules with glycolipids.

- ***Stratum lucidum*.** It has 2-3 cell layers and it is a thin clear layer consisting of eleidin, which is a transformation product of keratohyalin.
- ***Stratum corneum*.** It is the uppermost layer of the skin. It consists of 10-30 cell layers of corneocytes (terminally differentiated and fully cornified KCs). These cells are continuously replaced by KCs coming from the *stratum basale*. In addition, corneocyte layer not only secretes defensins but also provides mechanical protection to the rest of epidermal layers, and a barrier to avoid water loss and invasion by exogenous substances. Complete cell turnover happens every 28-30 d in young adults and every 45-50 d in elderly adults.

- **Dermis** is placed below the epidermis and is the thickest of the three layers that form the skin. The main role of this layer is to maintain the temperature and to provide the epidermis with nutrient-saturated blood. The dermis is made of fibrous, filamentous and amorphous connective tissue, and is divided into papillary and reticular sub-layers (2, 3, 5, 6).
  - **Papillary dermis** (upper layer) presents capillaries that transport nutrients and oxygen to the skin and remove cell products and waste. This sublayer contains a thin arrangement of collagen fibers and is in contact with the epidermis.
  - **Reticular dermis** (lower layer) is thicker and denser than the upper layer thanks to the concentration of collagen and elastic fibers.

  In both layers there are different types of cells such as fibroblasts, myofibroblasts and immune cells (macrophages, innate and adaptive lymphocytes and mast cells) (2).

- **Subcutaneous layer or hypodermis.** This is the deepest layer of the skin and presents a network of fat and collagen cells. For this reason, this layer can exert a relevant role in the regulation of the body temperature and in the protection of internal organs (3, 5).

Physical barriers. The corneocytes situated in the *stratum corneum* are responsible for the epidermal function as a barrier (2, 7). Moreover, junction adhesion molecules (JAMs) and tight junction proteins, such as claudins, zonula occludens-1 (ZO-1) and occludins are essential players in the establishment and maintenance of the physical barrier. Alterations in the function of these structural components promote an anomalous formation of the barrier, inflammatory conditions in the skin, and skin disorders such as atopic dermatitis (AD) (2, 8, 9).

Biomolecules. To defend the organism against exogenous agents, skin presents different biomolecules that can induce the disruption of bacterial membranes. The main biomolecules are antimicrobial peptides (AMPs) and lipids. AMPs are amphipathic peptides which can be expressed constitutively or induced after cell activation under inflammatory conditions. There are several groups of AMPs in the human skin including defensins, cathelicidins (CAMP), psoriasin and dermcidin. These biomolecules are secreted by different types of cells like KCs, fibroblasts, DCs, monocytes and macrophages. Besides, sweat and sebaceous glands also produce these AMPs (2, 10, 11).

pH. In contrast to other organs, the pH of the human skin is in the range from 5.4 to 5.9, which produces hostile conditions for possible pathogens. Changes in the pH are involved in different skin pathologies such as AD (2, 12, 13).

Immune and non-immune cells. Skin-resident immune cells not only promote the function of the tissue in homeostasis but also exert an important role as sentinels by scanning exogenous antigens. In a pathological situation, skin resident immune cells and infiltrated cells defend the organism and repair the tissue damage. In skin, there are resident myeloid cells such as LCs, dermal DCs, macrophages, mast cells and eosinophils, which participate in the maintenance of skin homeostasis by secreting growth factors and phagocytosing apoptotic cells. Moreover, in inflammatory conditions, these cells and neutrophils can produce pro-inflammatory mediators that induce the activation and migration of immune cells. On the other hand, skin shows a huge variety of resident non-immune cells such as KCs, fibroblasts, adipocytes, melanocytes and endothelial cells which express pattern recognition receptor (PRR). The activation of PRRs induces the secretion of cytokines, chemokines and AMPs that activate the local immune response, thus promoting the protection of the organism against external agents (2).

Both human and murine skin contain lymphoid immune cells such as γδ and αβ T cells, which exhibit a relevant role in both homeostasis and inflammatory responses. In contrast to humans, γδ T cells are the main T lymphocyte subset in some epithelial barriers such as mouse skin (2, 14). Once naïve T cells are activated by antigen presenting cells (APCs), some of them can differentiate into memory T cells such as central memory T cells, effector memory T cells or resident memory T cells (TRM) (15). αβ T cells are located in the epidermis and dermis, and they home to skin from the periphery. In the skin, they are established as a TRM cells and they are long-lived cells. Most TRM are derived from antigen-specific T cells, which previously infiltrated the tissue as a consequence of a damage or alteration such as inflammatory or infectious disorders (2, 16, 17). For example, pathogenic $CD8^+$ TRM cells are mainly found in the epidermis and can displace dendritic epidermal T cells (DETC) in mouse model of dermatitis (18). $CD4^+$ TRM are found in epidermis and dermis, and they establish clusters with antigen-presenting cells around the hair follicles that can circulate between the skin and periphery during homeostasis (19). Both $CD4^+$ and $CD8^+$ TRM cells play a relevant role in the cycle of relapsing-remitting skin disease (20, 21). Moreover, regulatory T cells (Treg) exert an important role in the resolution of the inflammatory diseases (2, 22).

Exacerbated expansion and activation of γδ T cells in the skin is a common feature of acute and chronic skin inflammation. Different γδ T subsets showing differential developmental and functional features are found in mouse and human skin (2, 23, 24). In mouse, most γδ T lymphocytes express CD27 and secrete interferon-γ (IFNγ) (γδIFNγ T cells), whereas interleukin- (IL-) 17 production is restricted to $CD27^-$ γδ T cells (γδ17 T cells) (25, 26). Moreover, mouse γδ T cells are divided into several subsets depending on their expression of Vγ chains, which is associated with their tissue tropism and effector function (27). Herein, we use the Tonegawa nomenclature to catalogue mouse Vγ chains (28). Mouse $CD27^+$ γδIFNγ T cells can express Vγ1 or Vγ4, while $CD27^-$ γδ17 T cells are Vγ4 or Vγ6 (27).

Different subtypes of γδ T cells are found in mouse skin in steady state, including DETC (Vγ5Vδ1) (29), and dermal resident Vγ6 and Vγ4 T cell populations, which secrete IL-17 (24, 30, 31). DETC account for 90 % of lymphocytes in mouse epidermis (32). DETC are sessile cells with a dendritic form that remain in close contact with surrounding KCs and LCs (33, 34). This population exclusively originates from the yolk sac at embryonic day E13.5 and persists throughout the life of the mouse by limited expansion in the epidermis (35). DETC are radio-resistant cells that recognize skin-derived self-antigens and participate in immune surveillance (36). At steady state, DETC turnover is slow, but following skin injury, clonal proliferation of tissue-resident cells supports their replenishment and homeostasis (35). Importantly, an equivalent population to DETC has not been found in human epidermis yet, and their frequency is variable in different mouse strains (37, 38).

Dermal Vγ6 and Vγ4 T cell populations predominantly express IL-17A and IL-22 (24, 31, 39). They share a very similar program of gene expression in steady state that includes phenotypic markers such as RORγt, CCR6, CD44 and CD69 (40). However, they differ in their origin, as well as in their functional requirements (41). Vγ6 T cells are generated solely in the thymic second wave around embryonic day E16, and exclusively express the Vδ1 TCR chain. On the other hand, Vγ4 T cells appear in the late fetal stage and newborn thymus and show variable Vδ partners. While dermal Vγ6 T cells are considered "bona fide" tissue resident that do not recirculate out of the skin (40), dermal Vγ4 T subset can migrate at low rate to lymph nodes in steady state (32, 39). The migration of Vγ4 T cells is enhanced during skin infection or during contact hypersensitivity reaction (CHS) (32, 39).

On the other hand, human γδ T cells can be divided into four main populations based on TCRδ chain expression (δ1, δ2, δ3, δ5). Vδ2 co-expressing the Vγ9 chain is the most frequent subset of γδ T cell population in the peripheral blood and secondary lymphoid organs of fetal and adult individuals. Vγ9Vδ2 T cells are developed in the fetal liver and thymus (42-44). Human $V\delta2^-$ γδ T cells are mainly represented by Vδ1 T cells and, to a lower extent, by Vδ3 and Vδ5 T cells. Vδ1 T cell subset is heterogeneous in the usage of Vγ chains and preferentially resides in epithelial tissues such as the skin and intestine (45).

According to the described protective skin component that is altered, **skin pathologies** are classified into three different groups (46):

- **Disorders of the barrier.** This group includes disorders in which components of the physical or chemical barrier are altered. For example, patients with AD present a decreased expression of claudin-1 (47) and filaggrin (13). In addition, changes in AMPs expression are related to the persistence of the AD (47).
- **Disorders of innate immunity.** These diseases include those innate immunodeficiencies related to alterations in the innate immune receptors (TLRs) (e.g. MyD88 deficiency) (48), innate immunohyperactivity disorders (e.g. acne and rosacea) and innate autoimmunity diseases (e.g. systemic lupus erythematosus, systemic sclerosis and psoriasis) (46).
- **Disorder of acquired immunity.** This group includes, for example, immunodeficiencies (e.g. Severe Combined Immune Deficiency (SCID)), allergies/immunohyperactivity (e.g. food allergies,

allergic contact dermatitis and irritant contact dermatitis) and autoimmune diseases (e.g. alopecia areata and psoriasis) (46).

1.2. CONTACT DERMATITIS

Skin diseases are very common disorders representing one of the most prevalent occupational diseases in the world. Taking into account all occupational diseases present in Europe, skin disorders represent more than 30 % (49, 50). **Contact dermatitis (CD)** is the most frequent occupational skin disease (95 %) induced by chemicals or metal ions which exert irritant effects, or by contact allergens that promote the activation of the immune system (51, 52). CD involves two types: **i) irritant contact dermatitis (ICD)**, a primary toxic skin reaction to chemicals or metal ions and **ii) allergic contact dermatitis (ACD)**, an immune reaction caused by the contact with a hapten **(Figure 1.1)** (51, 53). ACD is one of the most common disease of the skin that affects 15-20 % of the general population in the world (54, 55).

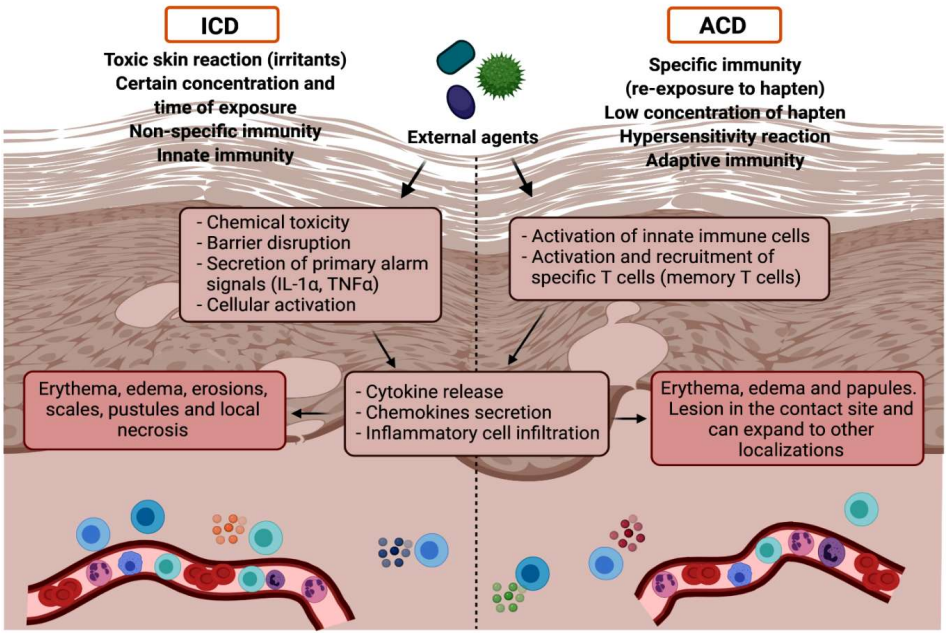

Figure 1.1. Scheme of the mechanisms involved in ICD and ACD. Created with BioRender.com

1.2.1. Irritant contact dermatitis

ICD is the most common type of CD and represents approximately 70-80 % of occupational CD cases (50, 56). For many years, it has been considered as a non-immunological disorder but, nowadays, it is known that innate immune cells play an important role in the development of ICD (50, 57). It is a non-specific skin response to chemical or physical agents, known as irritants, which induce the activation of the immune system without prior sensitization with them (49). These irritants are described as agents that can produce cellular alteration under specific concentrations and after sufficient time of exposure. Many common irritants are used daily like eye cosmetics, detergents, shampoo, metal tools, etc. Moreover, some environmental factors (e.g. temperature, humidity and airflow) or host-related factors (e.g. age, sex, genetic factors, etc.) are known to predispose the development of ICD (56). The typical clinical features of ICD are erythema, edema, erosions, scales, pustules and local necrosis **(Figure 1.1 and 1.2)** (51, 57). Besides, some patients develop a tolerance to repeated exposure to irritant agents (56).

The main players in the initiation of the immune response are the KCs. Thus, the first event in the development of this disorder is the skin barrier disruption promoting the response of resident cells to irritant agents. The secretion of primary alarm signals such as IL-1α and tumor necrosis factor-α (TNFα) causes the release of secondary mediators such as chemokines (CC-chemokine ligand 20 (CCL20) and C-X-C Motif Chemokine Ligand 8 (CXCL8)) (58) and growth factors, and induces the expression of adhesion molecules that promote the infiltration of leucocytes (e.g. neutrophils, lymphocytes and macrophages) into the skin damage (49, 50, 59). Moreover, anti-inflammatory cytokines such as IL-10 are produced in response to the exposure with the irritant, and they are involved in the resolution of the inflammation **(Figure 1.2)** (56).

1.2.2. Allergic contact dermatitis

ACD occurs after skin re-exposure to a specific hapten. It is a delayed hypersensitivity reaction (type IV) to allergens that promotes T cell response. In the sensitization phase, the first contact with the hapten takes place and this molecule penetrates the epidermis. In the second phase, known as elicitation, the re-exposure to the hapten occurs and specific αβ T cells are activated and recruited to the challenge site (56, 60-62). This pathology depends on the rapid activation of specific $CD4^+$ and $CD8^+$ T cells that secrete cytokines and chemokines (63, 64). In contrast to ICD, ACD requires lower concentration of the hapten to promote this pathology in sensitized patients. Furthermore, patients with ACD display the lesion in the contact site with the agent but, it can also expand in the focus or to other localizations. Classical lesions of ACD present similar features and may be difficult to differentiate from other eczematous processes as AD. In the acute phase of ACD, patients show erythema, edema and papules, and in the chronic stage of the disease, skin becomes lichenified, fissured and pigmented **(Figure 1.1 and 1.2)** (50, 51, 65). For patients with mild disease, the standard treatments are antihistamines and corticosteroids. Patients with severe ACD require a systemic steroid treatment and phototherapy (65).

Figure 1.2. Clinical manifestations of ICD (left) and ACD (right). Taken from (51).

1.2.3. Haptens

There is a large variety in the origin and nature of compounds that can induce an ACD disease. Contact allergens are low molecular weight chemicals usually under 500 Da (55). These small compounds cannot induce an immune response by themselves and they need to bind to epidermal proteins to generate new antigenic determinants and an adaptive immune response. In ACD, there are different types of contact allergens according to their nature: **i)** electrophilic molecules and sensitizing metal ions (e.g. nickel or cobalt) which bind directly to self-proteins, leading to hapten-protein complexes, and **ii)** non-electrophilic molecules which are transformed by the environment (i.e. oxidation or radiation) such as

terpene fragrances (linalool, D-limonene) or by skin enzymes promoting highly reactive metabolites such as fragrances (eugenol) or drugs (hydrocortisone) (55, 66). All of these are considered weak haptens and are the most frequent in humans (67).

1.2.4. Contact hypersensitivity model

CHS model is one of the most frequently used mouse models of ACD. It allows studying both phases of ACD, the sensitization and the elicitation. In this model, it is possible to assess tissue inflammation by measuring the ear swelling as direct readout of the immune system response to the hapten. There are different ways to induce CHS depending on the allergen applied. The most used contact allergens are: oxazolone (OXZ), fluorescein isothiocyanate (FITC), 2,4-dinitro-1-fluorobenzene (DNFB), 2,4,6-trinitrochlorobenzene (TNCB), ovalbumin (OVA) or co-application of nickel and Toll-like receptor (TLR) 4 ligand (53, 61). OXZ and DNFB are the main strong haptens used to induce CHS model and are able to sensitize more than 90 % of naïve individuals after a single skin contact (67, 68).

1.2.5. Pathophysiology of CHS

Sensitization phase. In this phase, the first contact with the hapten promotes the generation of hapten-specific T lymphocytes in the lymph nodes, which home to the skin. To establish this first phase, the full activation of DCs is critical. In the activation of these cells, both TLRs and cytosolic nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) are involved. The hapten induces the production of reactive oxygen species (ROS) that promote the chemical modification of the extracellular matrix. These matrix components like biglycan or hyaluronic acid act as activator ligands for TLR2/4 and induce a signaling cascade (MAPK (mitogen-activated protein kinase)/NF-κB (nuclear factor k-light-chain-enhancer of activated B cells)) to promote IL-1β and IL-18 production. Similarly, in relation to NLRs, KCs are activated by haptens through NLRP3, which controls the production of these proinflammatory cytokines. It is described that these cytokines participate in the migration and maturation of DCs, which are relevant to the establishment of the sensitization phase. In addition, NLRP3 can be activated by both hapten-induced intracellular adenosine triphosphate (ATP) and extracellular ATP secreted by damaged, stressed and dying cells, which interacts with purinergic receptors (P2X7) **(Figure 1.3a)** (55).

Therefore, during the sensitization phase, haptens act directly on KCs and mast cells or via innate immune system. These interactions promote the secretion of many mediators that activate DCs (55). KCs release IL-1α, IL-1β, TNFα, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-8, and IL-18, which induce vasodilation, cellular recruitment, and infiltration (59). Moreover, mast cells stimulate emigration of skin DCs to the lymph node (69). Activated LCs and dermal DCs (dDCs) capture antigens, maturate and migrate from the skin to the draining lymph nodes (dLNs) through afferent lymphatic vessels. Haptens are processed by DCs and are presented to naïve $CD8^+$ and $CD4^+$ T cells through the major histocompatibility complex-I (MHC-I) or MHC-II, respectively, in the dLNs. Activated specific clones differentiate and proliferate into effector and memory T cells that migrate from the lymph nodes

to the skin (55). $CD8^+$ TRM cells are mainly localized in the epidermis, whereas $CD4^+$ TRM cells are accumulated in the dermis (70). Generation of Treg suppresses the DC-mediated effector T cell generation **(Figure 1.3b).** This sensitization phase lasts 10-15 d in humans and 5-7 d in mice. This initial phase has no clinical symptoms in most cases but can promote a primary ACD characterized by skin inflammation in the contact site (55, 66).

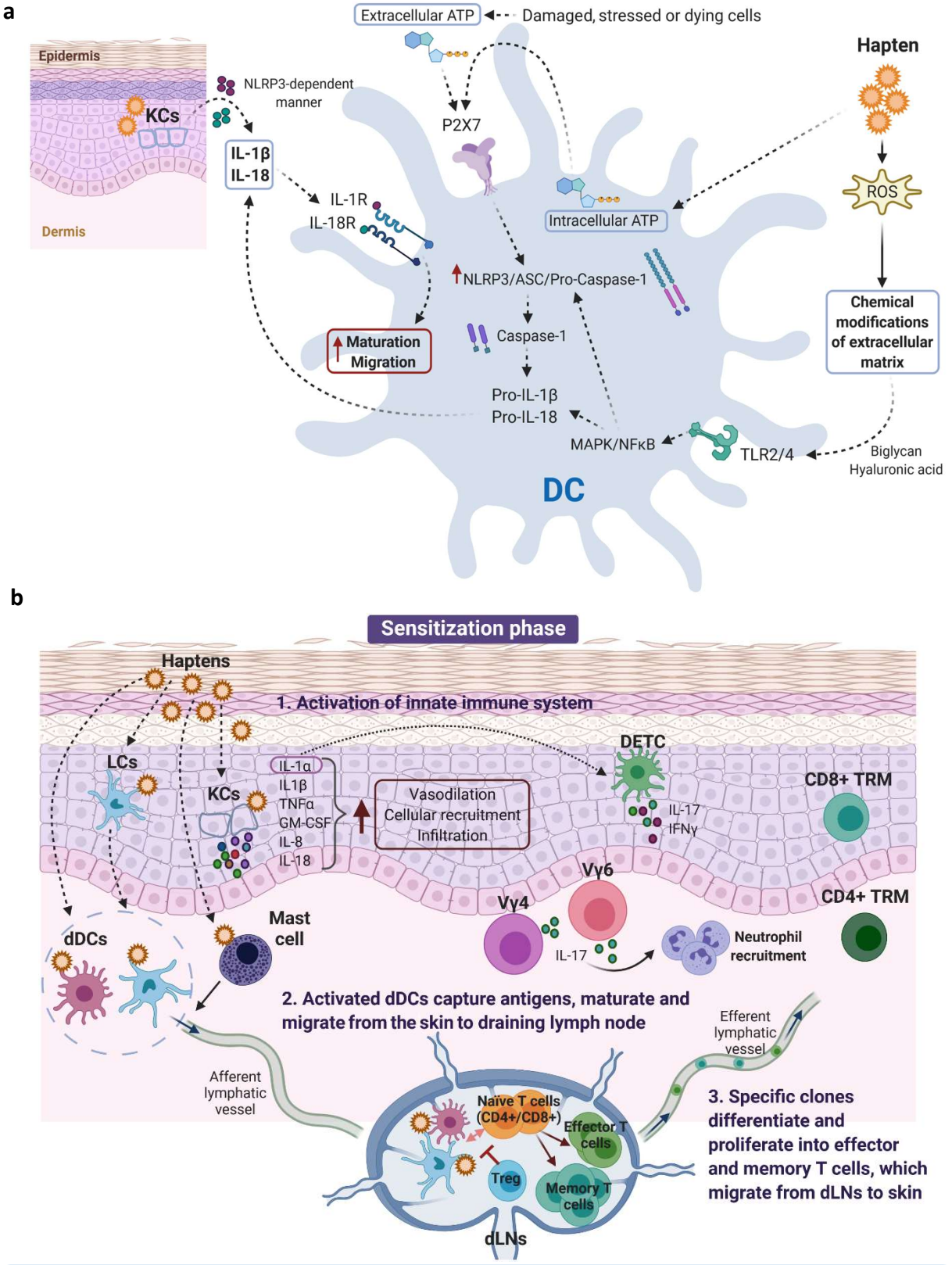

Figure 1.3. (a) Activation of DC by hapten. Modified from (55). **(b) Scheme of the mechanisms involved in sensitization phase.** Created with BioRender.com

Elicitation phase. It is characterized by the re-exposure of sensitized individuals to the same hapten. This second contact leads to the development of ACD in the next 24-72 h. After re-exposure, the hapten activates KCs and mast cells which secrete chemical mediators that activate endothelial cells to express adhesion molecules as ICAM-1 (intercellular adhesion molecule-1) and P/E-selectins. This activation promotes the infiltration of immune cells including neutrophils and memory T cells to the skin (55). Both $CD4^+$ and $CD8^+$ TRM cells participate in the re-exposure to the hapten, but long-term response is mediate mainly by $CD4^+$ TRM cells (20). Activated DCs present the antigen to antigen-specific effector T cells, which secrete pro-inflammatory cytokines (IFNγ and IL-17). Furthermore, these cytokines activate KCs promoting more infiltration of immune cells. On the other hand, Treg cells home to inflammatory sites to suppress the inflammation **(Figure 1.4)** (55). Therefore, the inflammatory response responsible for the cutaneous lesions is mediated by specific T lymphocyte activation in the skin. The elicitation phase lasts 72 h in humans. The inflammatory reaction persists for only a few days and rapidly dampens due to down-regulatory mechanisms (55, 66).

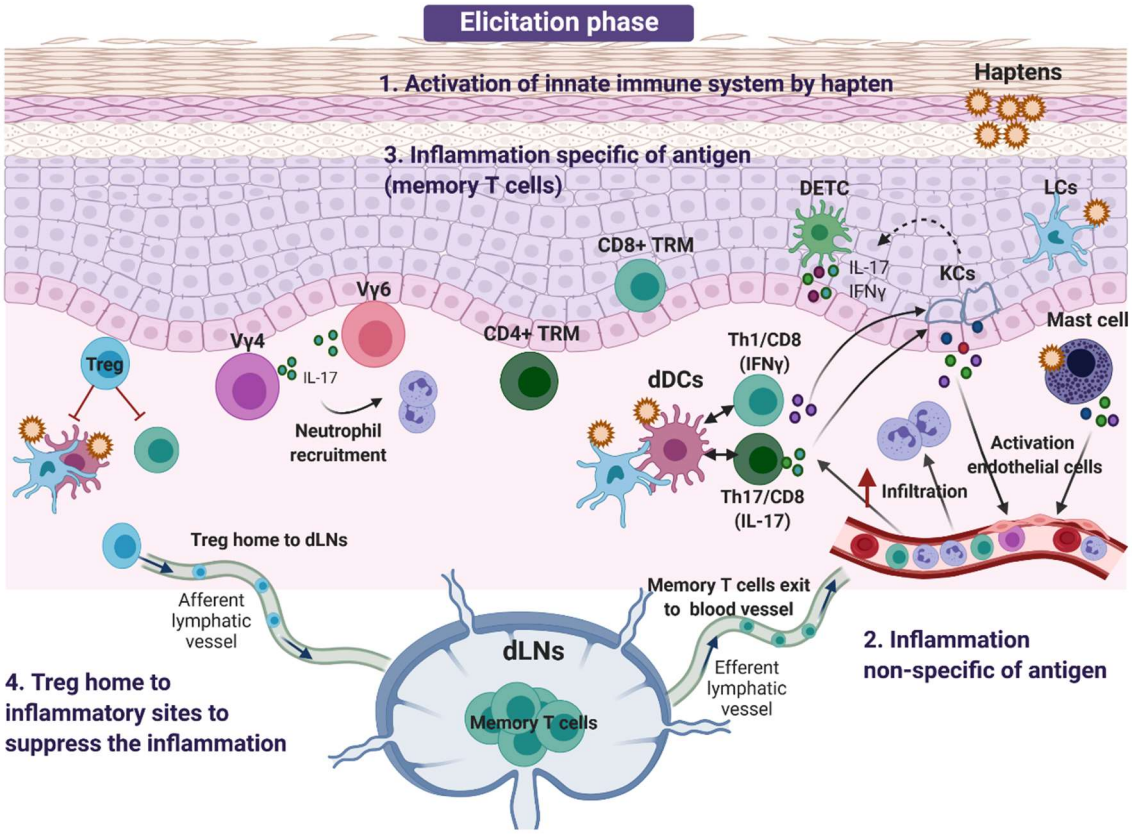

Figure 1.4. Scheme of the mechanisms involved in elicitation phase. Created with BioRender.com

In the development of CD not only αβ T cells but also γδ T cells and natural killer T cells (NKT) have a relevant role (71). Both $CD4^+$ and $CD8^+$ can act as effector T cells in the development of ACD (72). $CD8^+$ T cells are considered as the main effector cells to most of haptens in the development of CHS model, whereas $CD4^+$ T cells act mainly as downregulatory cells (68, 72). However, in absence of $CD8^+$ T cells, $CD4^+$ T cells can be effector cells of CHS. Furthermore, although it was historically considered a T helper 1 (Th1) or a mixed Th1/Th2 response, ACD is increasingly recognized as involving production

of Th17 and Th22 cytokines. Some sensitizers (e.g. fragrances) induce Th2 polarization, while others (e.g. nickel) generate a Th1/Th17 polarity (73). On the other hand, the putative role of γδ T cells in CHS model is controversial. Early studies showed that TCRδ$^{-/-}$ mice display increased CHS reaction due to γδ T cell-mediated regulation of specific CD8^{+} effector T lymphocyte development and their cytotoxic activity (74). On the other hand, some authors observed that TCRδ$^{-/-}$ mice display less inflammation in CHS model (39, 75).

DETC can migrate from the skin to dLN during CHS at very low rate, which is affected by deletion of occludin protein (76). Hapten-activated KCs can secrete IL-1β, which in combination with TCR activation, can promote DETC-release of IL-17 and IFNγ, thus promoting skin inflammation **(Figures 1.3b and 1.4)** (75). IL-1β signaling by DETC also controls their activation and migration to the lymph node after skin sensitization (75). DETC recognition of NKG2D ligands expressed by KCs is required for allergen-induced activation of DETC and IL-17 secretion induced by IL-1β (77). However, other studies have indicated a potential anti-inflammatory role of DETC in CHS models. TCRδ$^{-/-}$ mice in the FVB background are more susceptible to develop ACD and ICD reaction than TCRδ$^{-/-}$ mice in the C57BL6 background (78). Adoptive transfer assays demonstrate that the spontaneous dermatitis induced in TCRδ$^{-/-}$/FVB mice can be avoided by the transfer of selected Vγ5 T cells (78). Although DETC are clearly involved in CHS response, dermal γδ T cell subsets are the main source of IL-17 in the skin after hapten exposition (39). Both fractions, Vγ4 and Vγ6 T cells are rapidly increased after the hapten application. Depletion of Vγ4 T cells clearly reduces the CHS response (39). Thus, dermal γδ17 T cells play a relevant pro-inflammatory role in CHS response because they can promote the recruitment of neutrophils to hapten-treated skin, in an IL-17 dependent manner **(Figures 1.3b and 1.4)** (39). The relative contribution of dermal Vγ6 vs. Vγ4 T cells to the IL-17 secretion in CHS models has not been evaluated in depth.

In contrast to mouse investigations, there are few studies in humans related to γδ T cells and ACD. γδ T cells isolated from ACD biopsies of patients sensitized with corticosteroids secrete IL-4 (79). In ACD patients, an early and sustained increment in the number of both Vδ1 and Vγ9Vδ2 T cells, in the dermis and epidermis, is observed after the contact with an allergen. These results indicate that although γδ T cells may not be participating in the establishment of the disease, they may play relevant roles in the early reaction against allergens (80). In particular, allergic and irritant reactions to gold chloride, mercuric chloride and nickel in humans increase the frequency of Vγ9Vδ2 T cells (81, 82).

1.3. PSORIASIS

Psoriasis is a common chronic skin disorder with a global prevalence of 2-3 % (83-85). The progress of this pathology can be affected by diverse factors such as immune system, genetic, autoantigens and environmental factors. Psoriasis can appear at any age, but it is more frequent in two intervals independently of sex: 18-39 and 50-69 years (86). In this skin disorder both innate and adaptive immune systems play a critical role, being KCs, DCs and T cells the main players. This immune-mediated disease

is characterized by the presence of cutaneous and systemic manifestations and can be classified into many subtypes **(Figure 1.5)** (86-89):

- **Psoriasis vulgaris (plaque-type psoriasis).** It is the most common manifestation occurring in more than 80% of the cases (85). Its features are erythematous areas and focal plaques of inflamed and raised skin covered by scales. The commonly affected areas in patients with this subtype are knees, elbows, scalp, umbilicus and lumbar area.
- **Eruptive guttate psoriasis.** It is the second most common type (10 %) (85). Patients show small, tear-shaped and erythematosquamous papules. In most cases, this psoriasis subtype appears in childhood or young people and as a consequence of a streptococcal infection.
- **Inverse psoriasis.** It is characterized by the presence of erythematous plaques in body folds and commonly appears with few scales. Frequently, patients with inverse psoriasis can develop other subtypes at the same time.
- **Pustular psoriasis.** Patients with this subtype show sterile pustules on an erythematous base and scales. The commonly affected areas are hands and feet.
- **Erythrodermic psoriasis.** It is a severe manifestation and affects about 3 % of patients with psoriasis. It is characterized by extensive erythema which can reach more than 90 % of the body and provokes severe itching and pain.

Figure 1.5. Clinical manifestations of the different types of psoriasis: (a) Psoriasis vulgaris, (b) Eruptive guttate psoriasis, (c) Inverse psoriasis, (d) Pustular psoriasis, and (e) Erythrodermic psoriasis. Taken from (86).

Patients can develop multiple phenotypes and all these subtypes share many symptoms that include itching, burning and soreness. In addition, it is known that most of these subtypes of psoriasis have a cyclic evolution, meaning that they appear during weeks or months and then disappear for an undetermined period of time. In some cases, the disease goes into a remission. Apart from these critical clinical features, this disorder is associated with psychological burden, because the visible skin lesions can lead to negative reactions in others, decreasing the quality of life of psoriatic patients (89).

Psoriasis has been associated with many comorbidities being the most common the psoriatic arthritis (0.3-1 % of the global population). In addition, patients with severe psoriasis show more predisposition to suffer cardiovascular diseases such as myocardial infarction and psychiatric complications. Other psoriasis-associated comorbidities are autoimmune diseases, obesity, metabolic syndrome, liver diseases, etc. (89).

1.3.1. Physiopathology and mechanisms of psoriasis

Psoriasis is a multifactorial disease in which aberrant skin immune response is modified by the interaction between many genes and also with the environment. Regarding the influence of the genetic component in the development of psoriasis, it is described that more than 70 genes are associated with this disorder. Several psoriasis susceptibility loci have been described in many regions of different chromosomes. It is known that the major genetic determinant of psoriasis (*PSORS1*) is located in the MHC on the short arm of chromosome 6. The three candidates in this region are human leukocyte antigen-C (HLA-C), corneodesmosin, and α-helical coiled-coil rod (HCR). HLA-C gene is the strongest candidate for this disease and its allele HLA-Cw*0602 is considered to be the risk allele (85-87, 90). Additionally, several studies have demonstrated the presence of three autoantigens in psoriasis and their relevance in the development of this disease. These autoantigens are LL-37, phospholipase A_2 group IVD (PLA2G4D) and ADAMTSL5. These autoantigens are increased in psoriatic plaques and patients with moderate to severe manifestations present autoreactive $CD4^+$ or $CD8^+$ against them (87, 90, 91).

In relation to the mechanisms of psoriasis, susceptible individuals can develop psoriasis induced by infections, traumas or medications. In the initial phase, these external insults, like KC damage, promote the secretion of self-nucleotides that form complexes with AMPs. For example, LL-37, the most studied CAMP, exerts a relevant role in the protection against pathogens but, in psoriasis, it is overexpressed. It is produced by KCs and other immune cells in response to bacterial or viral infections or skin trauma. This complex CAMP/nucleic acid is protected from enzymatic degradation and can bind to TLR7 and 9 on the surface of plasmacytoid DCs (pDCs) (86, 87). This is an important autoimmune mechanism by which pDCs respond to self-DNA linked with AMP (91). Then, the antigen presentation by infiltrated pDCs promotes the clonal expansion of antigen-specific $CD8^+$ TRM cells in the dermis and the activation of naïve T cells in the dLNs. Activated $CD8^+$ T cells migrate into the epidermis where they interact with KCs though MHC-I and promote the secretion of cytokines, chemokines and innate immune mediators (86). All these factors promote the increase of inflammation and KCs proliferation. In addition, infiltrated pDCs promote the production of IFNs and then, the activation of myeloid DCs (mDCs). mDCs secrete more pro-inflammatory factors like IL-12, IL-23 and TNF which promote in turn the expansion of Th1, Th17 and Th22 and the release of their characteristic cytokines. IL-23 plus IL-1β induce the secretion of IL-17 by Th17. IL-17 binds to the IL-17 receptor (IL-17R) presented in the KCs and promotes the secretion of TNF and a chemotactic factor for T cells and DCs, CCL20. Moreover, IL-17 and other pro-inflammatory factors promote the secretion of defensins and chemokines that induce the recruitment of additional inflammatory cells into the lesion. Additionally, IL-22 secreted by Th22 cells participates in the establishment of the typical histological features of psoriasis patients (epidermal hyperplasia and acanthosis). Indeed, the endothelial cells play an important role because they express vascular endothelial growth factor receptors that induce vascular proliferation and the expression of adhesion molecules to promote the recruitment of inflammatory cells (mast cells, macrophages and polymorphonuclear leukocytes (PMNs)) into the skin injury **(Figure 1.6)** (86).

Therefore, the pro-inflammatory cytokines IL-23, IL-1β, and IL-17 exert important functions in patients with psoriasis, as well as in patients with other chronic inflammatory diseases (92-94). IL-23 stimulates the survival and proliferation of Th17 cells, whereas IL-1 receptor (IL-1R)-deficient mice do not properly display Th17 cells (95). However, the molecular mechanism by which IL-23 receptor (IL-23R) and IL-1R contribute to the development, survival, and expansion of IL-17 cells remains unclear. Importantly, IL-1β potentiates the effects of IL-23 by inducing expression of IL-23R in both Th17 and γδ T cells (95, 96).

Psoriasis patients display increased homing of Vγ9Vδ2 T cells from the blood to the skin (97). In addition, dermal Vγ9Vδ2 T cells found in psoriatic patients secreted more IFNγ, TNFα, and IL-17A, inducing the recruitment of blood immune cells (97). Importantly, psoriasis-targeted therapy reverted the decreased numbers of circulating Vγ9Vδ2 T cells, indicating its role in the disease (97). However, the role of Vδ1 populations in psoriatic patients has to be explored in depth. Further studies are also required to assess the potential role of IL-23 responder subset, $CD26^{hi}CD94^{lo}V\delta2^{+}$ T cells recently identified (98).

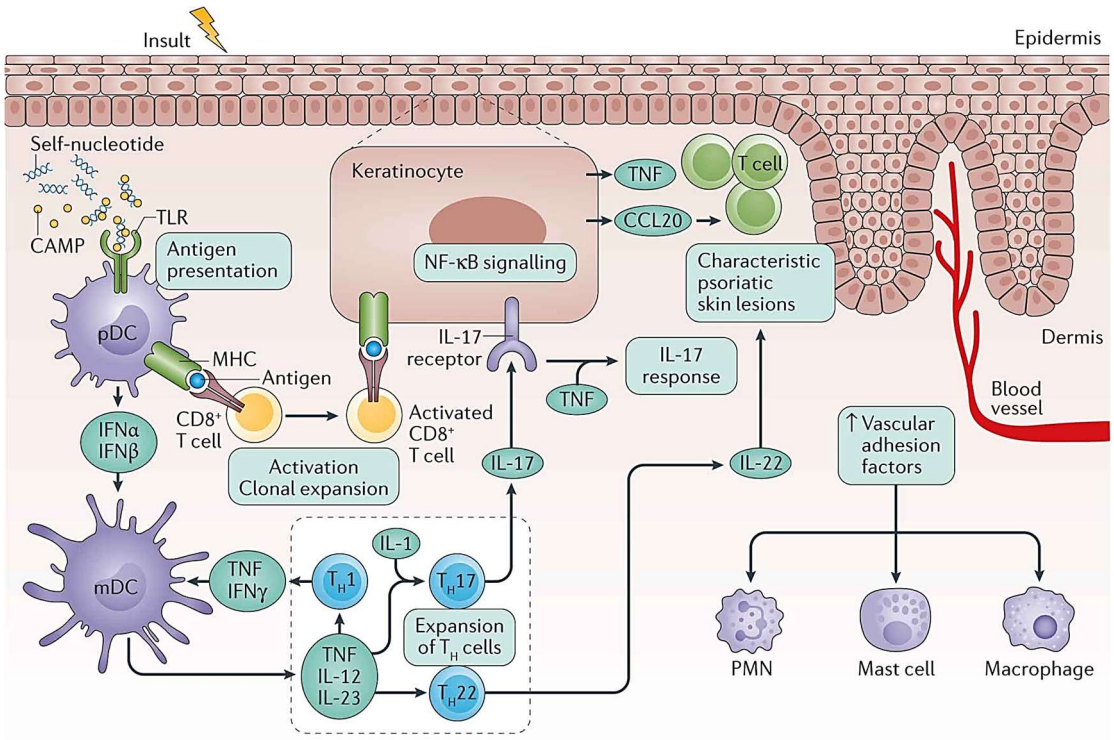

Figure 1.6. Scheme of the mechanisms involved in the development of psoriasis. Taken from (86).

1.3.2. Diagnosis and treatment

Most of the patients display cutaneous features and approximately 15 % of them present swelling or pain in the joints. There are different treatments depending on the extension of the disease and the existence of comorbidities. They can be divided into several groups **(Table A.1)** (85, 86, 99):

- **Topical therapy.** For patients with mild to moderate disease and without arthritis symptoms. For example: corticosteroids, vitamin D analogues, retinoids and calcineurin inhibitors. All of these are immune modulators.

- **Phototherapy.** For patients with moderate to severe disease and without arthritis symptoms. It is an effective treatment when the affected area is too large for topical applications. This therapy has different functions related to immune systems, such as promoting the apoptosis of inflammatory cells or increasing the secretion of anti-inflammatory cytokines.
- **Systemic treatments.** For patients with moderate to severe disease, especially when they present alterations in the face, scalps or palms or show psoriatic arthritis. For example:
    - Methotrexate: 5-aminoimidazole-4-carboxamide ribonucleotide transformylase inhibitor. It promotes the reduction of TNF, NF-κB, IL-17, IL-22, IL-23 and CCL20.
    - Acitretin: oral retinoid. It dampens the Th1 and Th17 activities and normalizes KC differentiation.
    - Cyclosporine: is a calcineurin inhibitor. It inhibits the activity of T cells.
    - Fumarates: downregulate TNF, IL-12 and IL-23 productions.
    - Biologic therapies such as anti-TNF, anti-IL-17, anti-IL-23 or anti-IL-12.

1.3.3. Mouse models of psoriasis

Because laboratory animals do not naturally develop psoriasis, research into the pathophysiology of this disease remains challenging. However, different animal models allow delving into the molecular mechanism involved in psoriasis (100). Although mouse skin differs from human skin in different aspects of the immune system, mouse models are typically used. Mice present $CD8^+$ DCs which are not found in humans (101). Moreover, mouse skin contains a large fraction of γδ T cells, whereas in human skin αβ T cells are the main population (14). DETC constitute the predominant T cell in mouse skin but they have not been identified in humans (37). In addition, there are differences in the balance of leukocyte subsets, TLRs, antibody subsets and defensins (14, 102). Thus, most of these mouse models mimic some but not all the symptoms observed in psoriatic patients (101, 103). Mouse models of psoriasis can be divided into different groups (101):

- **Spontaneous mouse models.** Many spontaneous mouse mutations that induce psoriasiform phenotypes have been described. For example, spontaneous mutation in C57BL/Ka mice (cpdm/cpdm) causes the development of chronic proliferative dermatitis characterized by redness, alopecia, scaling, and severe pruritus (104, 105).
- **Genetically modified mice.** In most of these models, increased expression or knockout of a specific gene is induced directly to the basal layer of the epidermis. For example: transgenic mouse with KCs expressing a constitutively active Stat3 by using a bovine KRT5 promoter presents skin lesions similarly to psoriasis (106).
- **T cell-based mouse model**. Immunodeficient mice (SCID) reconstituted with minor histocompatibility-mismatched naïve $CD4^+$ T cells develop similar lesions to patients (107).
- **Xenografts.** Non-lesional psoriatic skin or plaque of psoriasis is transplanted onto immunodeficient mice (108).

- **Induced mouse models.** Intradermal injections of IL-23 (109) and topical applications of Imiquimod (IMQ) (TLR7/8 agonist) (110) promote the development of skin inflammatory lesions similar to psoriasis. Therefore, these models are highly used to study the cellular and molecular mechanisms involved in this pathology in order to develop possible therapies. The relevance of the IL-23/IL-17 axis and γδ T cells in psoriasis has been proved in mouse models induced by IMQ or IL-23-intradermal injection (24, 111). However, both models present advantages and disadvantages (112). IMQ model is commonly used because IMQ-mediated skin lesions are similar to psoriatic plaque observed in patients. These lesions show increased epidermal proliferation, infiltrating cells and expression of IL-23, IL-1β, IL-22 and IL-17 (110, 113-115). Although IMQ model causes a huge inflammation only with the topical application, the mechanism of action not only depends on TLRs but also the vehicle of the IMQ cream can act independently of these receptors. Therefore, the response is very complex and not exclusive to TLRs signaling cascade (112). On the other hand, the response induced by IL-23 injection is based on the important role of IL23/IL-17A axis in the development of psoriasis (110, 116, 117). In contrast, the main disadvantage of this model is that the cytokine effect is limited because of the activation of a single pathway (112). Murine dermal γδ T cells are the main responders to IL-23 because they are the major population in the mouse skin expressing IL-23R in homeostasis (24).

  However, both models of IMQ and IL-23 injections show increased numbers of dermal macrophages, DCs, and KCs secreting IL-1β and IL-23 (24, 118, 119). Besides, fetal/newborn-derived Vγ4 and Vγ6 T cells, secreting IL-17 and IL-22, mediate skin inflammation in both models (41, 111). Although dermal Vγ6 T cells are clearly pathogenic, Vγ4 T cells are considered more relevant in IMQ model because they cluster both fetal/newborn-derived and bone-marrow-derived (inducible) populations (Vγ4Vδ4 T cells), showing increased production of IL-17 and marked expansion in the lymph nodes (41, 120, 121). However, whether a similar expansion of bone marrow-derived Vγ4 T cells is involved in the IL-23-psoriasis model has not been reported yet. The role of DETC in psoriasis models has been ruled out because these cells do not secrete IL-17 and IL-22 in the IMQ or IL-23-induced murine models (24, 39).

1.4. ROLE OF GAL-1 AND LAT1 IN SKIN DISEASES

1.4.1. Galectins

Galectins are β-galactoside-binding animal lectins expressed in many tissues and organs showing their highest expression in the immune system. Galectins are characterized by shared consensus amino acid sequences in the carbohydrate-recognition domain (CRD). CRD of galectins has approximately 130 amino acids but only some residues in this domain directly contact glycan ligands. In mammals, this family contains 15 members but four of them are not expressed in humans (Galectin-5, -6, -11 and -15) (122-124). Human galectins have been classified into three major groups **(Figure 1.7a)** (124):

1. **Prototypical galectins:** contain a single CRD that can form homodimers.

2. **Chimeric galectins:** display a single CRD and a large N-terminus domain.
3. **Tandem-repeat galectins:** present at least two different CRDs.

Galectins have different structural arrangements and some of them are active as monomers, but others require homodimerization such as Galectin-1 (Gal-1) (125, 126).

1.4.1.1. Galectin-1

Gal-1 was the first described galectin family member and its complete folding involves two anti-parallel β-sheets (125). This galectin is a homodimeric protein formed of 14.5 kDa subunits. This dimer is conserved by hydrophobic interaction at the monomeric interface and by the hydrophobic core (127, 128) **(Figure 1.7b)**.

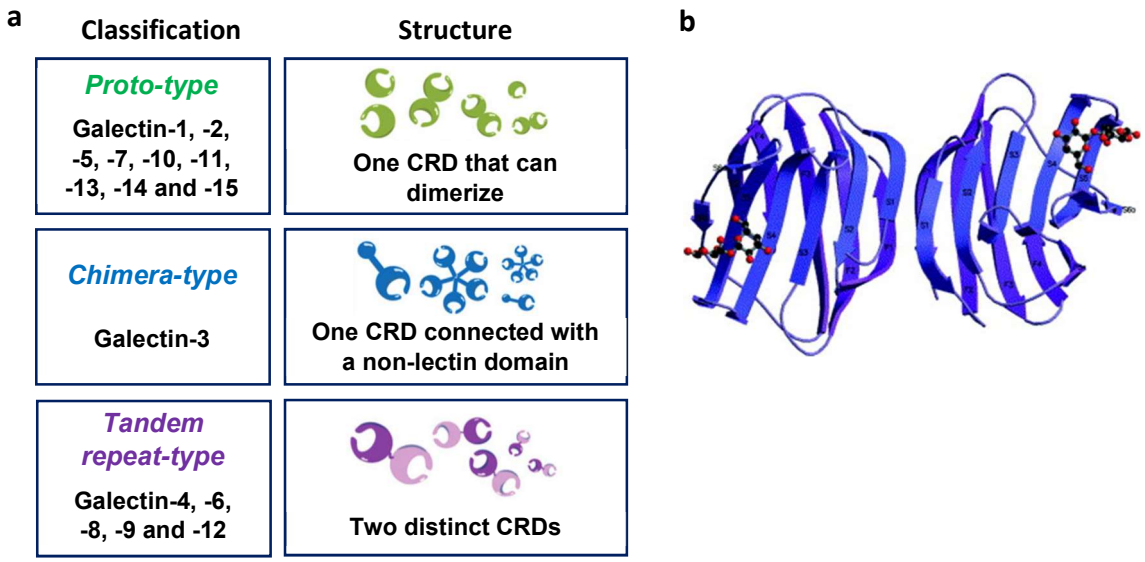

Figure 1.7. (a) Classification of Galectins. Modified from (122). **(b) Diagram of the homodimeric structure of Galectin-1.** Taken from (128).

Many normal and pathological tissues express Gal-1. In relation to the cellular localization, Gal-1 is detected inside (nucleus and cytosol) and outside of cells and exerts intracellular and extracellular functions. In the immune system, this lectin is synthesized and secreted by many different immune cell populations such as activated T and B cells, Treg, macrophages, DCs and γδ T cells (125, 129). Moreover, Gal-1 regulates different processes such as cell growth, migration (adhesion and motility) and nerve structural development (125, 129). Regarding the immune system, Gal-1 plays several functions related to:

- **T-cell homeostasis and survival.** Gal-1 induces the apoptosis of activated immune cells (130, 131) and inhibits cell growth (132). Moreover, the absence of Gal-1 in murine and human $CD4^{+}CD25^{+}$ T cells decreases their inhibitory capacity (133).
- **Tumor immune escape.** Gal-1 impairs the viability, proliferation, and Th1 responses of non-malignant T cells causing the progression of cutaneous T-cell lymphoma (134). In addition, Gal-1 has been described to induce the development of $IL\text{-}10^{+}$ $Foxp3^{-}$ regulatory cells, which are important players in tumor immune evasion (135).

- **Inflammation.** Gal-1-mediated anti-inflammatory properties have been demonstrated *in vitro* and *in vivo* experimental models. *In vitro* experiments with recombinant Gal-1 (rGal-1) demonstrated that it can inhibit the migration of human neutrophils (136). In *in vivo* ocular inflammation mouse model, rGal-1 has an anti-inflammatory role decreasing the migration of leukocytes into ocular tissues due to the imbalance in adhesion molecule expression (123). Moreover, in paw edema model, the administration of Gal-1 to wild-type (WT) mice decreases neutrophil recruitment, inhibiting the first phase of edema, similarly to the effects of endogenous Gal-1 (137).

1.4.1.2. CHS-Galectin-1

The role of Gal-1 in inflammatory skin diseases has been studied. For example, in OVA-induced AD model, rGal-1 treatment decreases the clinical signs and ear swelling associated with this inflammatory disease (63). Moreover, the anti-inflammatory role of Gal-1 has been explored in several *in vivo* and *in vitro* experiments in many pathologies including CHS (63, 123, 126, 136-138). Importantly, the administration of exogenous Gal-1 induces IL-10 release by $Foxp3^{-}CD4^{+}$ T cells, which suppress inflammation (126, 135). Moreover, the absence of Gal-1 expression in murine and human $CD4^{+}CD25^{+}$ T cells reduces their capacity to control proliferation of effector $CD4^{+}$ T cells (133). In OXZ-induced CHS, mouse Gal-1 human chimera administration dampens CHS response (126). However, the differential role of endogenous Gal-1 in the function of regulatory and effector T lymphocyte subsets in CHS has not been addressed. Due to the current limitations in ACD treatment, in this thesis work we have evaluated the mechanism by which Gal-1 has an important role as an anti-inflammatory agent in OXZ-mediated CHS model.

1.4.2. Amino acid transporters

The human organism shows different mechanisms to adapt its requirements to the available resources such as increasing or decreasing cell growth and proliferation. To modify these functions, cells have several nutrient receptors to obtain information about the intracellular and extracellular nutrient concentration. Many enzymes, receptors and transport proteins can act as sensors (139) and interact with specific cell nutrients such as amino acids, glucose, fatty acids, sterols and iron. These nutrients are cell signaling molecules and participate in the regulation of gene expression, protein phosphorylation cascade and endocrine mechanisms (140).

Amino acids are organic compounds that act as substrates for protein synthesis. Except for glycine, all amino acids present optical activity (141). Although L-amino acids are the main isoform that appears in physiology fluids, some of them may be shown in a D-isoform (142). To maintain the homeostasis in the organism, a tight balance between the intake of amino acids and the presence of them in the circulation is necessary. In mammals, there are 21 amino acids necessary for protein synthesis and are classified as follows (141, 143, 144):

- **Essential amino acids (EAAs)** are necessary to acquire in the nutrition because humans cannot synthesize them or are inadequately synthesized with respect to the need of the body. There are nine EAAs: histidine (His), leucine (Leu), isoleucine (Ile), valine (Val), phenylalanine (Phe), tryptophan (Trp), methionine (Met), threonine (Thr) and lysine (Lys).
- **Conditionally EAAs** can be synthesized by the organism but the diet should complement them to supply the requirements of the body in certain conditions, for example, arginine (Arg), glutamine (Gln) and tyrosine (Tyr).
- **Non-essential amino acids (NEAAs)** are not essential dietary nutrients but are a relevant energy source, for example, alanine (Ala), aspartic acid (Asp) and glutamic acid (Glu).

Moreover, it is well described that these molecules regulate critical cellular processes such as anabolism and catabolism depending on the amino acid availability (140). Besides, some amino acids participate in metabolic pathways involved in immunity, reproduction and growth (141). Because amino acids cannot diffuse across lipid membrane to exert these functions, they require membrane-transport proteins to access to the cell interior. This transport process requires different stages (143): **i)** amino acid binding to a specific site on the transporter; **ii)** conformational changes in the transport protein to exhibit the transported amino acid to the opposed face of the membrane; **iii)** release of the transported amino acid, and **iv)** reorientation of the transporter to the original position.

The amino acid binding region of the receptors can bind to many amino acids which share part of their structure, for example: large neutral AAs (LNAAs), small neutral AAs (SNAAs), cationic AAs (CAAs) or anionic AAs (AAAs) (139, 143). In cells, there are several amino acid transporters with overlapping specificities and therefore, the transport of one specific amino acid can involve the activation of other types of transporters. Moreover, the amino acid transport may depend on the simultaneous activation of different uniporters and antiporters, and it is sometimes linked to the transport of ions such as Na^+, H^+, K^+ and Cl^- (143).

Recently, based on the transporter gene sequence, the amino acid transporters are organized into 11 major families in the solute carrier (SLC) gene superfamily in humans (145, 146). SLC1, SLC6, SLC7, SLC16, SLC36, SLC38 and SLC43 are characterized by having multiple transmembrane domains (TMDs) **(Table A.2)**. Additionally, it is described that other amino acid transporters grouped into the SLC3 family present single TMD and exert an important function as regulatory subunits for SLC7 transporters. Moreover, SLC17 and SLC32 are vesicular transporters and SLC25 family members are involved in mitochondrial transport (139, 143, 146).

Among the amino acid transporters mentioned above, this thesis work is focused on those named as heterodimeric amino acid transporters (HATs), which have a heavy (SLC3 family) and a light (SLC7 family) subunit. SLC3 family presents two homologous heavy subunits rBAT and 4F2hc (also named as CD98) **(Figure 1.8)**. SLC7 family contains 15 members, two of them are pseudogenes, whereas the others encode proteins and can be divided in cationic amino acid transporters (CATs) and light subunits of amino

acid transporters, L-type amino acid transporters (LATs). Among these 13 SLC7 members, 9 light subunits of the HATs from SLC7A5 to SLC7A13 member are described **(Figure 1.8)** (147-149):

- Six of them are associated with 4F2hc: LAT1, LAT2, y+LAT1, y+LAT2, asc1, and xCT.
- One of them is associated with rBAT: $b^{0,+}$ AT.
- Two of them (asc2 and AGT-1) are partners of unknown heavy subunits.

The other 4 members (SLC7A1-SLC7A4) are system y^+ isoforms like cationic amino acids transporter and related proteins (147, 148).

Heavy Chain (HSHAT)	Light Chain (LSHAT)	Gene
4F2hc		*SLC3A2*
	y^+LAT1	*SLC7A7*
	y^+LAT2	*SLC7A6*
	LAT1	*SLC7A5*
	LAT2	*SLC7A8*
	asc1	*SLC7A10*
	xCT	*SLC7A11*
rBAT		*SLC3A1*
	$b^{0,+}$AT	*SLC7A9*
?		
	AGT1	*SLC7A13*
	asc2	*SCL7A12*

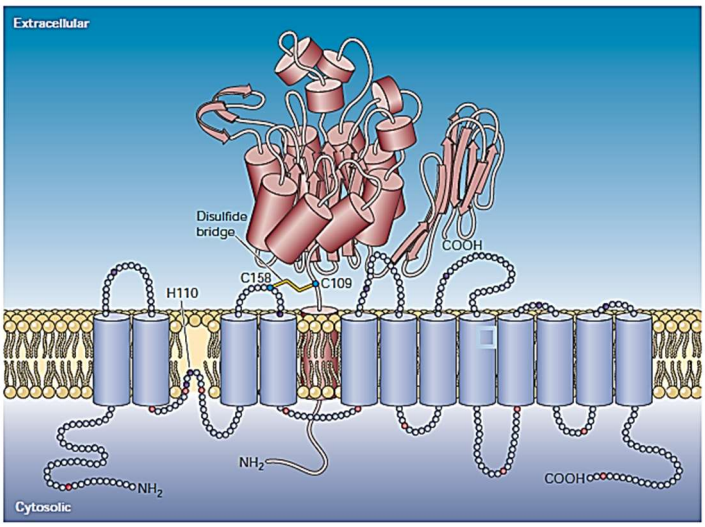

Figure 1.8. Heterodimeric amino acid transporters (HATs). List of members (left) and typical structure of the HATs (right). Modified from (147).

1.4.3. HAT: LAT1 / CD98 structure

As shown in **Table A.2** and **Figure 1.9**, LAT1 (SLC7A5) belongs to the SLC7 family and is a sodium- and pH-independent transmembrane transporter. To acquire the HAT conformation, this light subunit is associated with 4F2hc (CD98 or SLC3A2) through conserved disulfide bridge between C164 and C109 **(Figure 1.9)**. LAT1/CD98 complex imports LNAAs such as Leu and Phe and exports, for example, Gln being an antiporter system. Besides, this LNAA nutrient transport system is classically named as System L (148, 150).

LAT1 is a highly hydrophobic and not glycosylated polypeptide of 507 amino acids with a molecular weight of 55 kDa. LAT1 shows 12 α-transmembrane domains with both the N-terminus and the C-terminus localized intracellularly **(Figure 1.9)**. LAT1 is expressed in many locations such as testis, bone marrow, brain, placenta, basolateral membranes of polarized epithelia and apical and basolateral membranes of blood-brain barrier (148). On the other hand, the heavy subunit CD98 is a polypeptide of 630 amino acids with 70 kDa. This N-glycosylated protein has one transmembrane domain, an intracellular N-terminus and one large extracellular C-terminus **(Figure 1.9)** (150). CD98 is necessary for the stabilization and functional expression of LAT1 (151, 152). However, CD98 does not present transport activity and LAT1 is the unique transport competent subunit of the HAT (153).

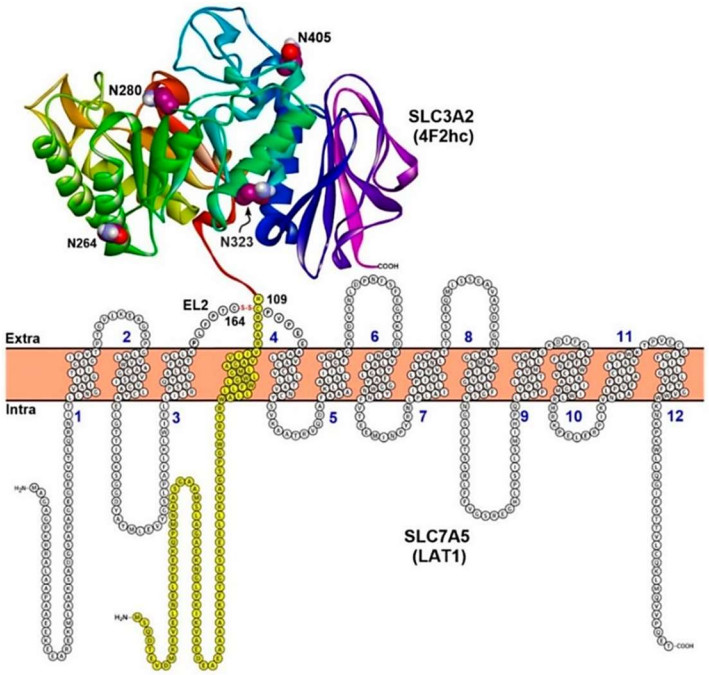

Figure 1.9. Structure of LAT1/CD98 complex. Taken from (150).

LAT1/CD98 mediates an antiport of Trp, Phe, Leu and His with high affinity and of Gln with less affinity. In contrast, Ala, proline (Pro) and charged amino acids are not transported by this HAT (148). Moreover, this complex can transport thyroid hormones and L-DOPA.

The alternative amino acid transporters LAT2/SLC7A8, LAT3/SLC43A1, and LAT4/SLC43A2 can also mediate LNAAs uptake (139). However, LAT1 is the main L-leucine (L-Leu) transporter expressed in activated T cells (154) and natural killer cells (NKs) (155). In addition, it is well described that LAT1 expression is involved in different diseases such as cancer, neurological disorders and blood-brain-barrier diseases (148, 156). The expression of LAT1 is increased in many cancer cells (157), including malignant skin lesions (158). LAT1 is the most important mediator of L-Leu uptake in malignant cells, and its usefulness as a target to inhibit cancer proliferation has been explored. Accordingly, the novel LAT1-specific inhibitor JPH203 has emerged as a promising agent for cancer therapy that blocks the phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway (159). In relation to blood-brain-barrier disorders, a decreased expression of LAT1 is correlated with the development of Parkinson's disease because of the reduction of L-DOPA (160). Moreover, LAT1 is involved in the normal neurological development through the L-tryptophan (L-Trp) transport by LAT1 (161).

1.4.4. LAT1-psoriasis

In relation to LAT1 and psoriasis, our group previously observed that CD69 regulates L-Trp uptake through its association with LAT1, which contributes to aryl hydrocarbon receptor (AHR) activation in skin-resident γδ T cells (162). In addition, LAT1 regulates L-Leu transport in $CD4^+$ T cells, controlling mTOR activation induced by TCR signaling (154). However, a direct contribution of LAT1 in the development of psoriasis *in vivo* remains unexplored.

OBJECTIVES

2. OBJECTIVES

Due to the large prevalence of inflammatory skin diseases worldwide, we aim to assess the effect of two immunoregulatory molecules, Gal-1 and LAT1, in allergic contact dermatitis and psoriasis, respectively.

- It is known that Gal-1 shows an anti-inflammatory role in several inflammatory diseases. Therefore, we postulate that endogenous Gal-1 may exert a regulatory role on the development of contact hypersensitivity induced by OXZ.

  In order to demonstrate this hypothesis, our main objectives are:

  I. Study the susceptibility of Gal-1 deficient mice to develop CHS response.
  II. Analyze Gal-1 expression by immune populations in CHS model.
  III. Study the role of circulating and endothelial Gal-1 expression in leukocyte migration in CHS model.
  IV. Assess the role of Gal-1 in the different populations of T cells in homeostasis, and in sensitization and elicitation phases of CHS model.

- Psoriasis risk correlates with alteration in metabolic pathways. Hence, we postulated that LAT1 expression in infiltrating lymphocytes and/or keratinocytes may play a relevant role in the development of psoriasis.

  In order to verify this hypothesis, our main objectives are:

  I. Analyze the LAT1 expression in keratinocytes and its function in psoriasis.
  II. Study the function of LAT1 in adaptive and innate lymphocytes and its role in IMQ-induced psoriasis.
  III. Ascertain the effect of pharmacologically inhibition of LAT1 in the psoriasis model.
  IV. Study the mechanisms through which LAT1 regulates the inflammatory response induced by IMQ.

OBJETIVOS

3. OBJETIVOS

Dada la gran prevalencia de las enfermedades inflamatorias de la piel, hemos analizado el efecto que tienen dos moléculas inmunoreguladoras, Gal-1 y LAT1, en el desarrollo de la dermatitis alérgica por contacto y la psoriasis, respectivamente.

- Se conoce que Gal-1 tiene un papel anti-inflamatorio en diversas enfermedades inflamatorias. Por tanto, postulamos que Gal-1 endógena podría estar ejerciendo un papel regulador en el desarrollo de la hipersensibilidad por contacto inducida por oxazolona.

  Para demonstrar esta hipótesis los objetivos principales planteados son:

  I. Estudiar la susceptibilidad que presentan los ratones deficientes en Gal-1 para desarrollar hipersensibilidad por contacto.
  II. Analizar la expresión de Gal-1 en las poblaciones del sistema inmune en el modelo de hipersensibilidad por contacto.
  III. Estudiar el papel de Gal-1 endotelial y circulante en la migración leucocitaria en el modelo de hipersensibilidad por contacto.
  IV. Evaluar el papel de Gal-1 en las diferentes poblaciones de células T en homeostasis y en las fases de sensibilización y elicitación del modelo de hipersensibilidad por contacto.

- El riesgo de desarrollar psoriasis se correlaciona con alteraciones en las rutas metabólicas. Por lo tanto, postulamos que la expresión de LAT1 en los linfocitos infiltrados y/o en los queratinocitos podría desempeñar un papel importante en el desarrollo de la psoriasis.

  Para verificar esta hipótesis los objetivos principales planteados son:

  I. Analizar la expresión de LAT1 en los queratinocitos y su función en la psoriasis.
  II. Estudiar la función de LAT1 en linfocitos innatos y adaptativos y su papel en el modelo de psoriasis inducida por IMQ.
  III. Evaluar el efecto de la inhibición farmacológica de LAT1 en el modelo de psoriasis.
  IV. Estudiar los mecanismos a través de los cuales LAT1 regula la respuesta inflamatoria inducida por IMQ.

MATERIALS AND METHODS

4. MATERIALS AND METHODS

4.1. CHEMICAL REAGENTS

OXZ (4-Ethoxymethylene-2-phenyl-2-oxazolin-5-one) was purchased from Sigma Aldrich (Missouri). The LAT1-specific inhibitor JPH203, (S)-2-amino-3-(4-((5-amino-2-phenylbenzo [d] oxazol-7-yl) methoxy)-3,5-dichlorophenyl) propanoic acid, was purchased from MedKoo Biosciences (North Carolina). All other reagents were purchased from Sigma Aldrich, unless otherwise indicated.

4.2. MICE

Mice were bred and maintained in the specific pathogen-free animal facilities of Centro Nacional de Investigaciones Cardiovasculares (Madrid, Spain). Experimental procedures were approved by the local Committee for Research Ethics and are in accordance with Spanish and European guidelines. Experiments were conducted with sex- and age-matched mice (8-12 weeks) kept on a regular 12 h light/dark cycle (7 a.m. - 7 p.m. light period), with food and water available *ad libitum*. All mouse strains used are on the C57BL/6 background.

- **Gal-1-CHS**

  Mice expressing IL-17-GFP and Foxp3-RFP proteins kindly provided by Richard A. Flavell's Laboratory (Yale University, USA) were backcrossed with WT (Gal-$1^{+/+}$) or Gal-1-deficient (Gal-$1^{-/-}$) mice (C57BL/6 background) (Jackson, Maine). In addition, $Rag1^{-/-}$ (C57BL/6 background) (Jackson) mice were backcrossed with Gal-$1^{+/+}$ and Gal-$1^{-/-}$ mice. CD45.1 C57BL/6 mice were purchased (Jackson).

- **LAT1-psoriasis**

  For all experiments, we used littermates derived from crossing Cre-negative $Slc7a5^{fl/fl}$ $tomato^{fl/wt}$ female mice with Cre-positive $Slc7a5^{fl/wt}$ $tomato^{fl/wt}$ male mice. $LAT1^{WT}$ mice are $Cre^{+/-}Slc7a5^{fl/wt}Tomato^{fl/wt}$ mice, and $LAT1^{\Delta}$ mice are $Cre^{+/-}Slc7a5^{fl/fl}Tomato^{fl/wt}$ mice. K5-CreERT2 mice were kindly provided by Erwin Wagner (Medical University, Austria). Mice (4 weeks) were fed with a 400-ppm tamoxifen diet (TD55125; Envigo, Indiana) for induction of Cre protein in the skin. $AHR^{-/-}$ mice were kindly provided by Prof. P. Salguero (Universidad de Extremadura, Spain). $AHR^{-/-}$ mice and their control littermate $AHR^{+/-}$ mice were obtained by breeding heterozygous parents. Mice expressing IL-17-GFP and Foxp3-RFP were used as WT mice for experiments with either JPH203 or rapamycin inhibitors.

4.3. HUMAN SUBJECTS

Patients with moderate-to-severe psoriasis who were recruited for the study had a Psoriasis Area and Severity Index of 8.0 or greater and washout periods of at least 14 d for topical corticosteroids and any systemic therapy. Skin punch biopsy specimens (3 mm) were obtained from patients with lesion plaque–type psoriasis and healthy volunteers. Blood samples (10 mL) were also collected from patients with

psoriasis and healthy volunteers. The study was approved by the Hospital Universitario de La Princesa ethics committee, and all participants provided written informed consent.

4.4. CONTACT HYPERSENSITIVITY MODEL

The shaved abdomen of mice was treated with 200 µL of OXZ solution (3 %) dissolved in ethanol at day 1. At day 5, a second challenge was induced using 20 µL OXZ (0.5 %) on both sides of the ear. As control, mice were painted with 20 µL vehicle alone (ethanol). Ear swelling, measured as increase of ear thickness, was assessed daily.

To carry out *in vivo* ICAM-1 blockade experiments, mice were treated with 150 µg of an anti-CD54 antibody (BioXCell; BE0020-1, New Hampshire) or the isotype control (BioXCell; BE0090) during 3 d after elicitation challenge.

$CD4^+$ T cells were depleted *in vivo* by using rat anti-mouse CD4 antibody (clone GK1.5; rIgG2b; BioXcell) (1 mg/mouse) injected intraperitoneally (i.p.) 24 h before and 48 h after the second challenge. Control mice were treated with rIgG2b isotype control (BioXcell). Complete depletion of $CD4^+$ T cells was confirmed by flow cytometry.

4.5. PSORIASIS MODEL INDUCTION AND TREATMENT

Induction of local psoriasis-like inflammation on ear skin was done through daily topical administration of 10 mg of IMQ cream (5 %) in each ear for 4 or 5 d. For systemic psoriasis induction, mice were mostly treated daily on shaved and depilated back skin with 50 mg of IMQ cream for 4 or 5 d. Exceptionally, $LAT1^{\Delta CD4}$ mice received 20 mg of IMQ daily on back skin for 8 d. When indicated, mice received daily doses of JPH203 (50 mg/kg of body weight) and rapamycin (5 mg/kg of body weight) dissolved in dimethyl sulfoxide (DMSO) (through the intraperitoneal route) during the course of IMQ treatment. Mice without IMQ or treatment application are always included as a control group. The IL-23 model of psoriasis was conducted as previously described (162). At least 10 intradermal injections of recombinant mouse IL-23 (500 ng in 20 µL of PBS; eBioscience, California) were performed per mouse on alternate days. When indicated, mice received an injection of 50 µL (250 µg) of Brefeldin A (Sigma Aldrich) dissolved in ethanol (5 mg/mL) and administered intraperitoneally 12 h before death. For *in vivo* bromodeoxyuridine (BrdU) labeling of cells, 1 mg of BrdU (BD Biosciences, California) dissolved in 100 µL of PBS was injected intraperitoneally 3 h before mice were killed.

4.6. ADOPTIVE TRANSFER EXPERIMENTS

$Gal\text{-}1^{+/+}$ mice treated with OXZ (3 %) were sacrificed at day 5 and T cell suspension were obtained from lymph nodes. $Rag1^{-/-}Gal\text{-}1^{-/-}$ or $Rag1^{-/-}Gal\text{-}1^{+/+}$ treated with OXZ in the ears were injected intravenously (i.v.) with the T cell suspension (1 donor : 1 recipient). Ears and lymph nodes were analyzed after 24 h. Similarly, $Gal\text{-}1^{+/+}$ and $Gal\text{-}1^{-/-}$ mice were treated with OXZ and T cell suspension from lymph

nodes were obtained and injected intravenously in CD45.1^{+}Gal-1$^{+/+}$ mice. Control mice were treated with vehicle.

CD4^{+} Foxp3^{+} (2.5 x 10^{6} cells/mouse), CD4^{+} Foxp3^{-} (50 x 10^{6} cells/mouse) T cells, and CD8^{+} cells (40 x 10^{6} cells/mouse) were purified (StemCell, Canada) and sorted for Treg separation (FACSAria Cell Sorter, BD Biosciences). Purified cells from OXZ-treated Gal-1$^{+/+}$ and Gal-1$^{-/-}$ mice were injected (i.v.) to CD45.1^{+} Gal-1$^{+/+}$ mice treated with OXZ in the ears. Control mice were injected with equal number of T cells from non-sensitized mice.

4.7. CHIMERIC MICE

CD45.1 mice (Gal-1$^{+/+}$) were lethally irradiated and transplanted with 5 x 10^{6} Gal-1$^{+/+}$ or Gal-1$^{-/-}$ bone marrow cells. After reconstitution (60 d), mice were challenged with OXZ as described above.

4.8. FLUORESCEIN ISOTHIOCYANATE (FITC) SKIN PAINTING

To test the migration ability of DCs in mice in the sensitization phase, mice were treated in the abdomen with 3 % OXZ and then, with FITC (10 mg/mL dissolved in acetone) (Isomer 1, Sigma Aldrich). Control mice were treated only with FITC. Migratory DCs from dLNs were analyzed by flow cytometry at 24 h, 48 h and 72 h.

4.9. SKIN HISTOLOGY AND IMMUNOHISTOCHEMISTRY STAINING

For histologic analysis, paraformaldehyde-fixed, paraffin-embedded skin sections were prepared and stained with hematoxylin and eosin (H&E). At least 3 skin sections (3-5 µm) 300 µm apart from each other were analyzed per mouse. Consecutive images were acquired at several magnifications with an optical microscope (DM2500; Leica) equipped with a CCD camera (DFC420; Leica), with Leica Application Suite software (version 4.3.0). For quantification of epidermal thickness, at least 10 measurements, randomly performed between all sections, were averaged per mouse. Similarly, ear samples were fixed in formaldehyde and embedded in paraffin. Slices were stained with H&E and digitalized. Epidermis and dermis thickness were measured every 100 µm. The measurements were performed using NDP Viewer software (Hamamatsu, Japan).

For immunohistochemical staining, skin sections were deparaffinized, boiled in the suggested antigen retrieval solution, and incubated with the primary antibodies indicated in **Table 4.1.** Slides were developed with diaminobenzidine substrate (K3468; Dako, Denmark) and then counterstained with Mayer hematoxylin.

4.10. IMMUNOFLUORESCENCE IN SKIN SECTIONS

Mouse skin sections were deparaffinized and boiled in antigen retrieval solution (Tris-EDTA buffer: 10 mM Tris base, 1 mM EDTA solution, 0.05 % Tween 20, pH 9.0). Skin sections were blocked in PBS

containing 5 % of chicken serum and were incubated with primary antibodies **(Table 4.1)** followed by specific secondary antibodies: chicken anti-rabbit Alexa Fluor 488 and chicken anti-goat Alexa Fluor 647. Nuclei were counterstained with DAPI. For immunofluorescence of LAT1 in mice, fresh skin fragments were embedded in OCT compound. IL-17-GFP was detectable in unfixed skin sections without staining.

Human skin sections were deparaffinized, boiled in the suggested antigen retrieval solution, blocked with BSA solution (2 %), and incubated with the primary antibodies indicated in **Table 4.1** for 18 h at 4 °C. The secondary antibodies used were the EnVision FLEX system for immunohistochemistry detection of LAT1/LAT2 (Dako) and Alexa Fluor 647-labeled chicken anti-rabbit for LAT1/LAT2 and LAT3 immunofluorescences. Nuclei were counterstained with 4′-6-diamidino-2-phenylindole dihydrochloride.

Images were captured with a Zeiss LSM 700 Confocal microscope and analyzed with LSM image browser software (Zeiss, Germany).

4.11. LYMPHOCYTES AND SKIN CELL PREPARATIONS FOR FLOW CYTOMETRY

Animals were euthanized and tissues were dissected and grated through a nylon mesh (70-µm; BD Biosciences) to obtain single-cell suspensions from lymph nodes, spleen and thymus. RBC lysis was performed with BD Pharm Lyse Buffer (BD Biosciences). Ears were separated into inner and outer halves, and digested with the following mix: Liberase TM (Roche, Germany) (0.08 mg/mL), collagenase IV (Sigma Aldrich) (0.5 mg/mL) and DNAse (Sigma Aldrich) (100 µg/mL) in RPMI medium supplemented with FBS (1 %) for 35 min at 37 °C. Enzymes were inhibited by adding 50 mL of PBS supplemented with 0.5 % BSA and 0.05 mM EDTA. Tissue was mechanically disrupted using 7-mm stainless steel beads (Life Technologies, New York) in a TissueLyser LT (Qiagen, Germany), one 3-min cycle (20 osc/sec). Isolated skin cells were flowed through a 70-µm nylon filter (BD Biosciences). For analysis of KCs, dorsal skin from mice treated or not with IMQ was incubated with 4 U/mL Dispase II (Roche) in MEM medium by 24 h at 4 °C.

4.12. FLOW CYTOMETRY

Single-cell suspensions from tissues and blood were incubated with anti-FcRII/III (clone 2.4G2) before staining and subsequently stained with the appropriated surface marker antibodies detailed in **Table 4.1.** Intracellular staining was conducted with the Fixation/Permeabilization Solution Kit (BD Biosciences). The staining panels always included dead cell staining with Fixable Yellow Viability Dye (Molecular Probes, Oregon). Absolute count of cells in the skin, spleen, lymph nodes and thymus was conducted using BD Trucount Tubes (BD Biosciences). BrdU detection was performed according to the manufacturer's protocol (BD PharMingen). Phospho-S6 ribosomal protein (Ser235/236) (P-S6) and Ki-67 staining were conducted with the Foxp3 Transcription Factor Staining Buffer Set (eBioscience) and with directed labeled antibodies against Ki-67 (B56; BD Biosciences) and pS6 (D57.2.2E; Cell Signaling, Massachusetts). For LAT1 and LAT2 detection, cells were fixed and permeabilized with Cytofix/Cytoperm

Kit (BD Biosciences), and Alexa Fluor 647-labeled chicken anti-rabbit was used as a secondary antibody. A customized rabbit anti-mouse LAT2 (kindly provided by Prof. M. Palacín, IRB, Universidad de Barcelona, Spain) was used. After staining, cells were washed and analyzed with FACSCanto or LSRFortessa (BD Biosciences). Data were further analyzed with FlowJo10 software (TreeStar, Oregon).

4.13. *IN VITRO* CULTURES AND CYTOKINE DETECTION

Mouse cells were cultured in RPMI or Iscove modified Dulbecco medium supplemented with FBS (5 %), 25 mmol/L HEPES, antibiotics, sodium pyruvate, and β-mercaptoethanol. When indicated, cells were incubated in RPMI 1640 medium without amino acids and supplemented with normal FBS (5 %; United States Biological, Massachusetts). Naïve $CD4^+$ T cells were purified from lymph nodes by using commercial kits (StemCell).

Naïve $CD4^+$ T cells were seeded (1×10^6 cells/mL) in a 24-well plate coated with 5 µg/mL anti-CD3 (145 2C11; Tonbo, California) for 48 h to test LAT1 expression and amino acid uptake experiments. For *in vitro* differentiation studies, naïve $CD4^+$ T cells were seeded (1×10^6 cells/mL) in supplemented Iscove modified Dulbecco medium in 24-well plates coated with 5 µg/mL anti-CD3 (145 2C11) and 1 µg/mL anti-CD28 (37.51) antibodies (Tonbo) and steered toward the Th17 lineage with IL-6 (50 ng/mL), IL-23 (10 ng/mL), IL-1β (10 ng/mL), and TGF-β (transforming growth factor beta) (1 ng/mL) for 4 d. When indicated, the Th17 cells were cultured with IL-6 and TGF-β for 4 d at concentrations indicated previously, and stimulation with IL-23 and IL-1β was tested only by 24 h.

Total $CD4^+$ T cells purified from $LAT1^{WT}$ and $LAT1^{\Delta CD4}$ mice after IMQ application were incubated by 18 to 24 h with IL-23 and IL-1β cytokines (10 ng/mL each) to stimulate secretion of cytokines and P-S6 induction or 48 h to assess proliferation. Similarly, cell suspensions from lymph nodes of $LAT1^{WT}$ and $LAT1^{\Delta R\gamma t}$ mice after IMQ application were split into 2 fractions: one was subjected to γδ T-cell depletion using anti-TCRγδ (clone GL3), and the other was used to purify γδ T cells with the EasySep Mouse Selection Kit (StemCell). The resulting fractions (purified γδ and γδ depleted) were *in vitro* stimulated by 18 to 24 h with IL-23 and IL-1β to assess cytokine release by means of ELISA. Ear cell suspensions after IMQ application were also *in vitro* stimulated with IL-23 and IL-1β to assess cytokine release. Supernatants were collected, and IL-17 and IL-22 levels were assessed by using mouse ELISA Ready-SET-Go Kits (Fisher Scientific, Massachusetts). $CD4^+$ T cells were further stimulated with phorbol 12-myristate 13-acetate (PMA) (50 ng/mL; Sigma Aldrich), ionomycin (1 mg/mL; Sigma Aldrich), and brefeldin A (GolgiStop, 1 µg/mL; BD Biosciences) at 37 °C in a 10 % CO_2 atmosphere for 4 h. After staining of surface markers, cells were fixed and permeabilized (Cytofix/Cytoperm and Perm/Wash Buffer; BD Biosciences), followed by staining with mAbs to mouse IL-17A and IL-22 (eBioscience). True count beads (BD Biosciences) were added to *in vitro* cultures to quantify the total number of expanded γδ T cells. Rapamycin (1 µmol/L), LY294002 (10 µmol/L), and JPH203 (10 µmol/L) inhibitors were added to the cultures when indicated. When indicated, BrdU at a final concentration of 5 µmol/L was added to assess proliferation according to the instructions of the BrdU Flow Kits (BD Biosciences).

PBMCs from healthy donors were obtained, and $CD4^+$ T cells were isolated by using the EasySep Human CD4 T Cell Isolation Kit (StemCell). For Th17 differentiation, isolated $CD4^+$ T cells were cultured for 12 d in RPMI 1640 medium supplemented with FBS (5 %), 25 mmol/L HEPES, antibiotics, and sodium pyruvate with anti-CD3 (5 µg/mL; catalog no. 300314, RRID:AB314050; BioLegend) plus anti-CD28 mAbs (2 µg/mL; catalog no. 555725; BD Biosciences). The following combination of cytokines and blocking antibodies was appropriate for Th17 polarization: rhIL-6 and IL-1β (10 ng/mL), rhIL-23 (20 ng/mL), rhTGF-β1 (2 ng/mL), anti-IFNγ (10 µg/mL), and anti-IL-4 (10 µg/mL; all cytokines from R&D Systems, Minnesota). These cytokines were added each 48 h. JPH203 at 10 µmol/L or DMSO was also added to the culture on alternate days. At day 12, cells were further stimulated by 4 h with PMA (50 ng/mL; Sigma Aldrich) and ionomycin (1 mg/mL; Sigma Aldrich) in the presence of brefeldin A (GolgiStop, 1 µg/mL; BD Biosciences). Fixed and permeabilized cells (Cytofix/Cytoperm and Perm/Wash Buffer; BD Biosciences) were stained with anti-IL-17 (BL168) and anti-IFNγ (B27) antibodies (BioLegend) and analyzed in a FACScanto cytometer.

For expansion of human γδ T cells, total human PBMCs (1×10^6 cells/mL) from heathy donors (buffy coats) were stimulated in a 24-well plate in culture medium EX-VIVO 15 (Lonza, Belgium) and expanded with zoledronic acid (5 µmol/L), as previously reported (163). At day 2, IL-2 (100 U/mL) and JPH203 inhibitor (10 µmol/L) were added and then replaced every 48 h. At day 10, the number of $Ki\text{-}67^+CD27^-$ γδ T cells was analyzed in a FACSCanto cytometer. LAT1 expression associated with CD98 was also confirmed in expanded (10 d) human γδ T cells by means of Western blotting.

4.14. *IN VITRO* ASSAY OF TREG FUNCTION

Mouse cells were cultured in RPMI medium supplemented with FBS (5 %), 25 mmol/L HEPES, antibiotics, sodium pyruvate, and β-mercaptoethanol. Single-cell suspensions of lymph nodes from OXZ-treated Gal-$1^{+/+}$ and Gal-$1^{-/-}$ mice were obtained. Treg cells were isolated and purify as $CD25^+$ T cells using EasySep™ Release Mouse Biotin Positive Selection Kit (StemCell). From the negative fraction ($CD25^-$ T cells), the $CD8^+$ T cells were purified (StemCell) and labeled using CellTrace Violet Cell Proliferation Kit (Thermo Fisher, Massachusetts). Cells were seeded (10 Treg : 1 CD8) in plate coated with 2 µg/mL anti-CD3 (145 2C11) and 0.5 µg/mL anti-CD28 (37.51) (Tonbo). $CD8^+$ T cells proliferation was assessed at 60 h.

4.15. RNA EXTRACTION AND QUANTITATIVE PCR ANALYSIS

Tissue total RNA was isolated with TRI Reagent (Sigma) or the Qiagen RNeasy Kit (Qiagen). Residual DNA contamination was removed with the Turbo DNA-free Kit (Ambion, Thermo Fisher). Total RNA (200-1000 ng) was reverse transcribed to cDNA with a Reverse Transcription Kit (Applied Biosystems, California). Quantitative PCR was then performed in an AB7900_384 (Applied Biosystems) by using SYBR Green (Applied Biosystems) as a reporter. Gene-specific primers used are listed in **Table**

4.2. Expression of each gene of interest was normalized to at least 2 housekeeping genes: β-actin (ACTB) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Data are presented as averaged relative fold differences calculated by using the $2^{-\Delta\Delta Ct}$ method with average values of healthy mice as a reference.

4.16. WESTERN BLOTTING

$CD4^+$ T cells from $LAT1^{\Delta R\gamma t}$ and $LAT1^{\Delta CD4}$ mouse cell lines were cultured (1×10^6 cells/mL) in RPMI medium for 24 h in the presence of anti-CD3 (5 µg/mL). After lysis with RIPA buffer supplemented with protease and phosphatase inhibitor cocktails (Roche), lysates were separated by using SDS-PAGE and immunoblotted with anti-LAT1 antiserum (kindly provided by Dr P. Taylor, Dundee, United Kingdom) (139). The loading control was carried out with a rabbit anti-mouse SMC protein 1A antibody (A300-055A; Bethyl Laboratories, Texas).

γδ T cells from PBMCs of patients with psoriasis were purified with the EasySep Human Gamma Delta T Cell Isolation Kit (StemCell) after 7-10 d in culture with zoledronate. $CD4^+$ T cells purified from PBMCs of patients with psoriasis or healthy volunteers were stimulated with anti-CD3 (2 µg/mL), anti-CD28 (1 µg/mL), IL-23 (20 ng/mL), and IL-1β (20 ng/mL) for 48 h. Lysates of human γδ T cells and $CD4^+$ T cells were done with RIPA buffer, separated by using SDS-PAGE, and immunoblotted with rabbit anti-LAT1 (5347S; Cell Signaling Technology) and mouse anti-β-actin (47778; Santa Cruz Biotechnology, Texas) or mouse anti-α-tubulin (T6199; Sigma Aldrich) as a loading control. Detection of LAT1, LAT2, and CD98 in cell lines (HaCaT, Caco-2, HeLa, and J77) was also conducted in fresh lysates prepared with supplemented RIPA buffer and with the antibodies indicated in **Table 4.1.** All primary antibodies were detected with horseradish peroxidase–conjugated goat anti-rabbit (Pierce, Illinois). Protein bands were analyzed with the LAS-3000 CCD system and Image Gauge 3.4 software (Fuji Photo Film, Japan).

4.17. QUANTITATIVE PROFILE OF L-LEU BY USING LIQUID CHROMATOGRAPHY–TANDEM MASS SPECTROMETRY

Serum L-Leu profiles of WT and immunocompromised ($Rag1^{-/-}$) mice treated or not with IMQ (for 5 d, 50 mg/d) were measured by using liquid chromatography–tandem mass spectrometry. Serum samples were obtained from at least 20 animals per genotype (10 healthy control mice and 10 mice with psoriasis) after 60 min of coagulation at 4 °C and were immediately stored at −80 °C until use. Stock solutions of leucine (Sigma Aldrich) and 13C11-Trp (Cambridge Isotope Laboratories, Massachusetts) were prepared in water (LC/MS grade) at 1000 ppm and used as an external standard and an internal standard, respectively.

4.18. AMINO ACID UPTAKE ASSAYS

Naïve $CD4^+$ T cells obtained from $LAT1^{WT}$, $LAT1^{\Delta R\gamma t}$, and $LAT1^{\Delta CD4}$ mice were cultured (1×10^6 cells/mL) in RPMI medium for 24 h in the presence of anti-CD3 (5 µg/mL) and used to test amino acid uptake in the presence of LAT1 inhibitors. The 3H-radiolabeled amino acids L-phenylalanine and L-Leu

(PerkinElmer, Massachusetts) were added (0.5 µCi/ml) in HBSS (Gibco, California) at a final extracellular L-Leu concentration of 5 µmol/L. Amino acid uptake was measured at 60 min at 37 °C. Incubation with the LAT1 inhibitors JPH203 (10 µmol/L) and 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (40 mmol/L) was done 10 min before addition of radioactivity. Uptake was stopped by addition of 20 mmol/L cold L-Leu to quench L-System. At the end of the assay period, cells were harvested onto glass-fiber filters using a Tomtec 96-well parallel harvester (Tomtec, Connecticut). B-radioactivity was counted in a Beckman LS 6500 Multi-Purpose Scintillation Counter (Beckman Coulter, California). At least 6 replicates were assessed for each data point.

4.19. STATISTICAL ANALYSIS

Statistical evaluations were performed with GraphPad Prism 7 software (GraphPad Software, California). After analysis of data distribution with Kolmogorov-Smirnov test, the statistical significance for comparison of 2 populations was assessed by 2-tailed Student's t test or Mann-Whitney test. Multiple comparisons were performed by using 1-way ANOVA or 2-way ANOVA with Bonferroni multiple-comparisons post hoc test, as required. Differences were considered significant at $P < 0.05$.

Table 4.1. List of used antibodies

Specificity	Reactivity	Clone	Fluorochrome	Origin	Dilution	Catalog No
CCR6	Mouse	29-2L17	BV421	BioLegend	1:200	129818
CD3e	Mouse	145-2C11	APC / BV421 / Purified	BD Biociences / Tonbo	1:200 / 1:250	553066 / 561416 / 70-0031
CD4	Mouse	RM4-5	APC / BV421 / Biotin	Tonbo / BD Biociences	1:200	20-0042 / 740007 / 553045
CD8	Mouse	53-6.7	APC FireTM750 / Pe-Cy7	BioLegend / Tonbo	1:200	100766 / 60-0081
CD25	Mouse	PC61.5	Biotin	Tonbo	1:200	30-0251
CD27	Mouse	LG.7F9	Pe-Cy7	eBiocience	1:200	25-0271-82
CD28	Mouse	37.51	Purified	Tonbo	1/1000	70-0281
CD45	Mouse	Polyclonal Goat IgG	Purified	R&D Systems	1:40	AF114-SP
CD45.2	Mouse	104	Pe-Cy7	eBiocience	1:200	560696
CD11b	Mouse	M1/70	FITC / Biotin	BD Biociences	1:200	553310 / 553309
CD11c	Mouse	HL3	PE / Biotin	BD Biociences	1:200	557401 / 553800
CD64	Mouse	X54-5/7.1	APC	BioLegend	1:200	139311
CD98	Mouse / Human-Mouse	RL388 / H300	Alexa Fluor 647 / Purified	BioLegend / Santa Cruz Biotechnology	1:200 / 1:500	128210 / sc-9160
EOMES	Mouse	Dan11mag	PE	eBiocience	1:200	12-4875-80
FcRII/III (CD16/CD32)	-	2.4G2	Purified	TONBO	1:100	70-0161
Gal-1	Mouse	D608T / Polyclonal Rabbit IgG	Biotinylated / Purified, IHC formulated	R&D Systems / Cell Signaling	1:100	BAF1245 / 13888
GITR	Mouse	DTA-1	Pe-Cy7	BioLegend	1:200	126317
Granzyme B	Mouse	NGZB	Pe-Cy7	eBiocience	1:200	25-8898-80
IFNγ	Mouse	XMG1.2	FITC / APC	eBiocience	1:200	11-7311-82 / 17-7311-82
IL-4	Mouse	11B11	PE	Biolegend	1:200	504104

IL-10	Mouse	JES5-16E3	BV421	Biolegend	1:200	505022
IL-17A	Mouse	TC11-18H10	PE	BD Biociences	1:100	559502
IL-22	Mouse	IL22JOP	APC	eBiocience	1:100	17-7222-82
Ki-67	Mouse	B56	APCcy7	BD Biociences	1:100	558615
LAT1	Mouse	H-75	Purified	Santa Cruz Biotechnology	1:50	sc-134994
Ly6C	Mouse	AL-21	PerCP	BD Biociences	1:200	560525
Ly6G	Mouse	1A8	Pe	BD Biociences	1:200	551461
PD-1	Mouse	29F.1A12	BV421	Biolegend	1:200	135217
pS6 (Ser235/236)	Mouse	D57.2.2E	Purified	Cell Signaling	1:100	8520S
T-bet	Mouse	4B10	PerCP/Cy5.5	eBiocience	1:200	45-5825-82
TCRαβ	Mouse	H57-597	APC-Cy7	BioLegend	1:200	109220
TCRγδ	Mouse	GL3	PerCP/Cy5.5 / Biotin	eBiocience	1:200	560696 / 13-5711-82
TCR-Vδ4	Mouse	GL2	PE	BioLegend	1:200	134905
TCR-Vγ4	Mouse	UC3-10A6	APC	BioLegend	1:200	137704
SLC7A5	Human	Polyclonal	Purified	Sigma Aldrich	1:200	HPA052673
SLC7A8	Human-Mouse	Polyclonal	Purified	Sigma Aldrich	1:200	HPA051950
SLC43A1	Human	Polyclonal	Purified	Sigma Aldrich	1:200	HPA01882

Table 4.2. Sequences of used primers

Gene	*Specie*	*Forward primer*	*Reverse primer*
Actb	Mouse	CAGAAGGAGATTACTGCTCTGGCT	TACTCCTGCTTGCTGATCCACATC
Ccl20	Mouse	ACTGTTGCCTCTCGTACATACA	GAGGAGGTTCACAGCCCTTTT
Ccr6	Mouse	ATGCGGTCAACTTTAACTGTGG	CCCGGAAAGATTTGGTTGCCT
Gapdh	Mouse	AGCTTGTCATCAACGGGAAG	TTTGATGTTAGTGGGGTCTCG
Hprt	Mouse	GCAGTACAGCCCCAAAATGG	GGTCCTTTTCACCAGCAAGCT
Ifng	Mouse	ATGAACGCTACACACTGCATC	CCATCCTTTTGCCAGTTCCT
Il10	Mouse	GCTCTTACTGACTGGCATGAC	CGCAGCTCTAGGAGCATGTG
Il17a	Mouse	TTTAACTCCCTTGGCGCAAAA	CTTTCCCTCCGCATTGACAC
Il22	Mouse	ATGAGTTTTTCCCTTATGGGGAC	GCTGGAAGTTGGACACCTCAA
Il23	Mouse	ATGCTGGATTGCAGAGCAGTA	ACGGGGCACATTATTTTTAGTCT
S100a8	Mouse	AAATCACCATGCCCTCTACAAG	CCCACTTTTATCACCATCGCAA
S100a9	Mouse	ATACTCTAGGAAGGAAGGACACC	TCCATGATGTCATTTATGAGGGC
Slc3a2	Mouse	GACACCGAAGTGGACATGAAA	GCTCCTCCTTGGATAAGCCG
Slc7a5	Mouse	CTGGATCGAGCTGCTCATC	GTTCACAGCTGTGAGGAGC

RESULTS

5. RESULTS

Due to the complexity and prevalence of the inflammatory skin diseases objects of this thesis work, we analyze the role of two molecules, Gal-1 and LAT1, as potential targets in ACD and in psoriasis, respectively.

5.1. REGULATORY ROLE OF ENDOGENOUS GAL-1 IN THE PROGRESSION OF CHS

5.1.1. Gal-1$^{-/-}$ mice show increased ear swelling and inflammation after CHS

To assess the role of endogenous Gal-1 in the regulation of lymphocyte functions in CHS model, OXZ was administered to Gal-1$^{+/+}$ and Gal-1$^{-/-}$ mice **(Figure 5.1a)**. Gal-1$^{-/-}$ mice showed increased ear swelling and sustained inflammation compared to Gal-1$^{+/+}$ mice **(Figure 5.1b)**.

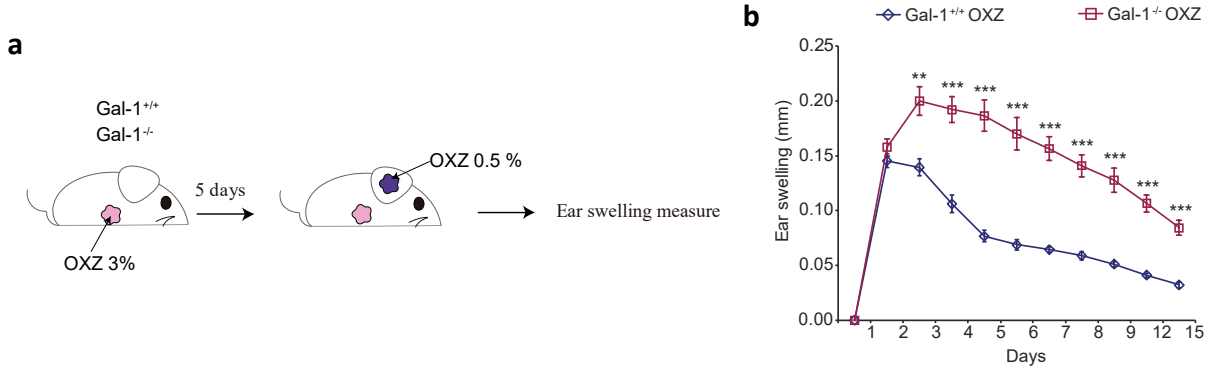

Figure 5.1. Gal-1$^{-/-}$ mice display increased ear swelling after CHS challenge. (a) CHS model diagram. **(b)** Ear swelling in Gal-1$^{-/-}$ and Gal-1$^{+/+}$ mice after OXZ. Results are expressed as the increase in ear thickness with respect to untreated ear day by day. Individual data (mean ± SD) from one representative experiment of three were shown. **P < 0.01, and ***P < 0.001, 2-way ANOVA with the Bonferroni post hoc test.

Histological analysis revealed that the increase in epidermal and dermal thickness was higher in Gal-1$^{-/-}$ than in Gal-1$^{+/+}$ mice at 48 h after the elicitation phase **(Figure 5.2)**.

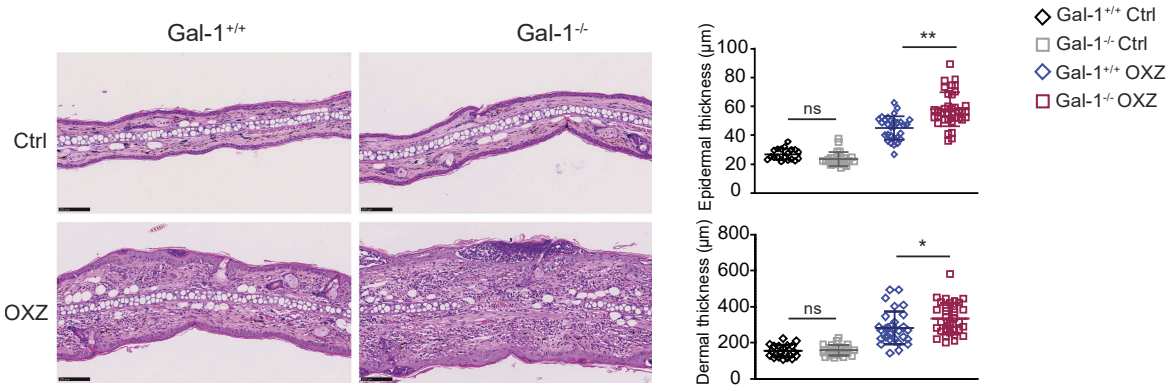

Figure 5.2. Gal-1$^{-/-}$ mice display increased skin thickness after CHS challenge. H&E-sections of OXZ-treated Gal-1$^{+/+}$ and Gal-1$^{-/-}$ ear mice (48 h) **(left)**. Epidermal/dermal thickness values **(right)**. Scale bars, 100 µm. Individual data (mean ± SD) from one representative experiment of three were shown. ns, not significant; *P < 0.05 and **P < 0.01, 1-way ANOVA with the Bonferroni post hoc test.

Furthermore, flow cytometry analysis showed that OXZ significantly increases the total numbers of infiltrating CD45^{+} cells and myeloid cells such as neutrophils (CD11b^{+} Ly6G^{+}) and macrophages (CD11b^{+}

CD64$^+$) in Gal-1$^{-/-}$ compared to Gal-1$^{+/+}$ mice **(Figure 5.3)**. These results suggest that the expression of Gal-1 prevents the inflammation induced by OXZ.

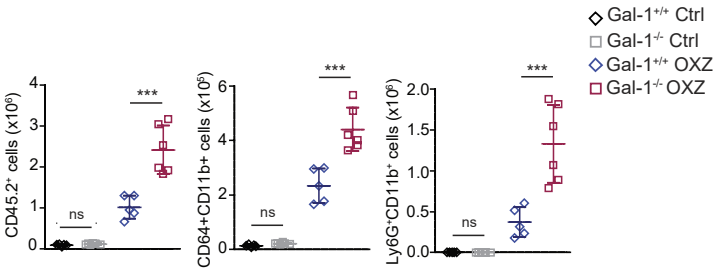

Figure 5.3. Gal-1$^{-/-}$ mice display increased inflammation after CHS. Total skin CD45$^+$ cells, neutrophils (Ly6G$^+$CD11b$^+$) and macrophages (CD64$^+$CD11b$^+$) were quantified by flow cytometry. Individual data (mean ± SD) from one representative experiment of three were shown. ***P < 0.001, 1-way ANOVA with the Bonferroni post hoc test.

We analyzed whether the expression of Gal-1 is differently modulated in lymphoid cells, compared with blood and skin tissue, upon CHS challenge. The expression of Gal-1 among effector T cells (CD4$^+$, CD8$^+$, γδ$^+$) and regulatory T cells (CD4$^+$Foxp3$^+$) populations was higher in blood than in lymph nodes at 48 h **(Figure 5.4)**. However, the highest expression of Gal-1 was detected in T cells in the inflamed tissue **(Figure 5.4)**.

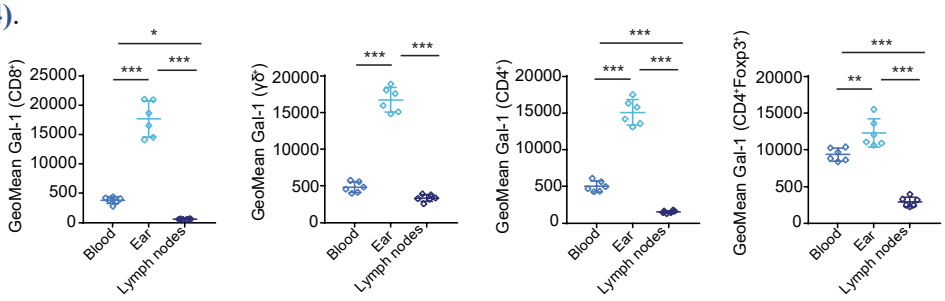

Figure 5.4. Gal-1 expression in different T cell populations detected in lymphoid and non-lymphoid organs. Gal-1 expression in CD8$^+$, γδ$^+$, CD4$^+$ and CD4$^+$ Foxp3$^+$ T cells after 48 h of OXZ treatment are shown. Individual data (mean ± SD) from one representative experiment of three were shown. *P < 0.05, **P < 0.01 and ***P < 0.001, 1-way ANOVA with the Bonferroni post hoc test.

We also evaluated Gal-1 expression in ear sections of Gal-1$^{+/+}$ mice treated with OXZ at 48 h after the second challenge by immunofluorescence. The detection of Gal-1$^+$ cells corresponds mainly to CD45$^+$ cells, although expression of Gal-1 was also observed in vessels, but not in KCs **(Figure 5.5)**.

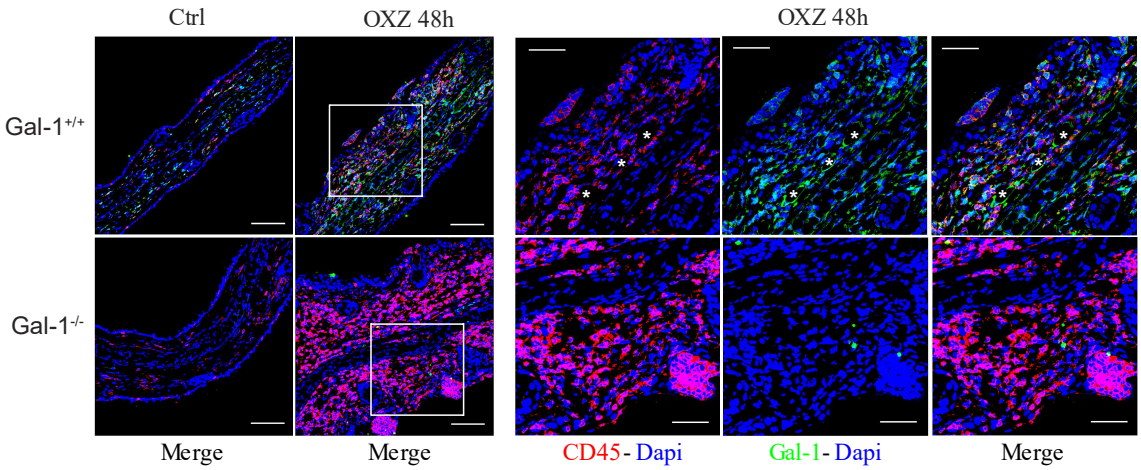

Figure 5.5. Gal-1 expression in ear after OXZ treatment. Immunofluorescence analysis of Gal-1 (green) and CD45 (red) in ear skin sections from Gal-1$^{+/+}$ and Gal-1$^{-/-}$ mice. Nuclei were always stained with DAPI (blue). Vessels are indicated with white asterisk. Zoom areas **(right)** are indicated by white boxes **(left)**. Scale bars = 100 and 50 μm in zoom areas. At least three skin biopsy specimens of mouse were analyzed.

5.1.2. Gal-1 deficiency increases skin effector T lymphocytes after elicitation phase of CHS

We analyzed the different T cell subsets in Gal-1$^{+/+}$ and Gal-1$^{-/-}$ mice expressing IL-17-GFP and Foxp3^{+} RFP protein, in steady state. These experiments demonstrated that Gal-1$^{-/-}$ mice are similar to Gal-1$^{+/+}$ mice in terms of total number and frequency of T cells populations (CD8^{+}, CD4^{+} and CD4^{+}Foxp3^{+}) in blood, skin, lymph nodes and spleen **(Figure 5.6)**. In the thymus, the absence of Gal-1 did not cause any significant difference in the number of CD4^{+}CD8^{+} T cells, single positive cells, or natural Treg cells (CD4^{+}Foxp3^{+} cells) **(Figure 5.7)**.

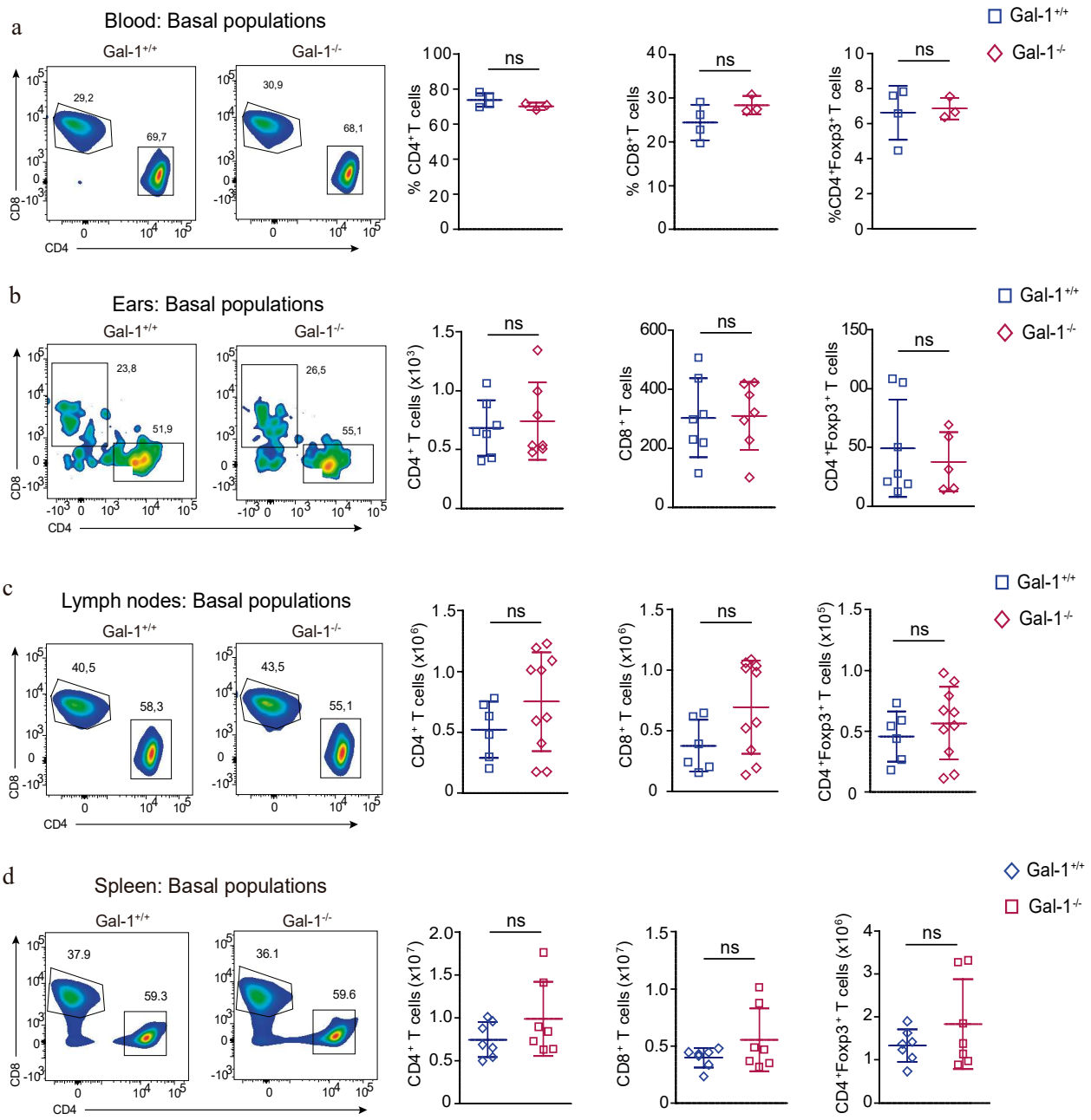

Figure 5.6. Gal-1 genetic deletion does not alter populations of CD4^{+} and CD8^{+} T cells in homeostasis. **(a)** Density plots **(left)** and frequency **(right)** of CD4^{+}, CD8^{+} and CD4^{+}Foxp3^{+} cells from TCRαβ^{+} gated cells in blood. Density plots **(left)** and total numbers of CD4^{+}, CD8^{+} and CD4^{+}Foxp3^{+} **(right)** from TCRαβ^{+} cells in ears **(b)**, lymph nodes **(c)** and spleen **(d)** in homeostasis in Gal-1$^{-/-}$ and Gal-1$^{+/+}$ mice. Data from one representative experiment of three are shown (mean ± SD). ns, not significant; 2-tailed unpaired Student t test analysis.

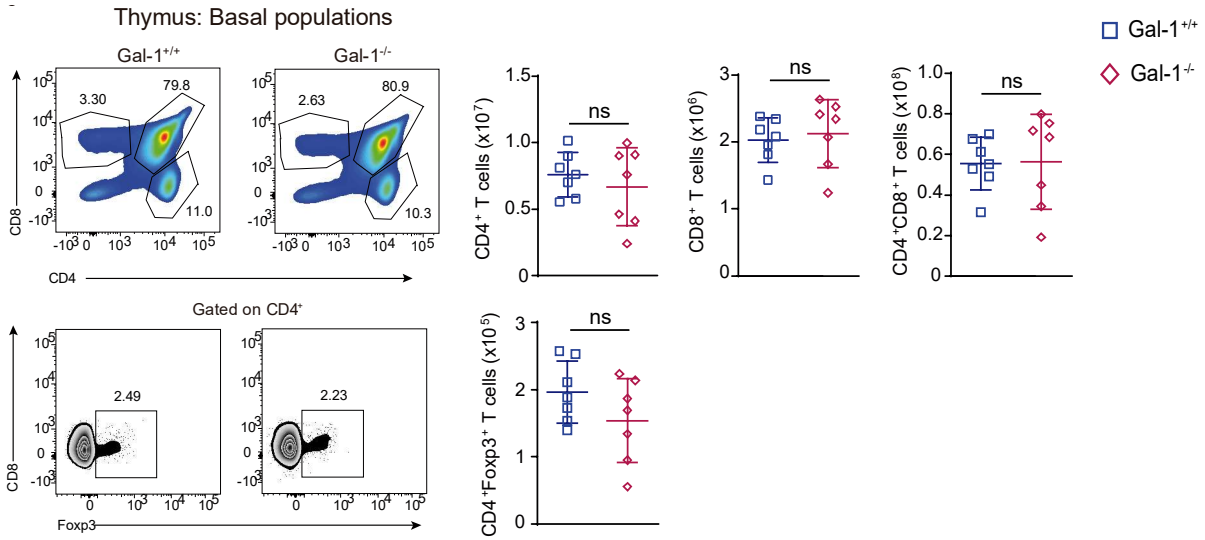

Figure 5.7. The absence of Gal-1 does not alter thymic populations of T cells in homeostasis. Density plots **(left)** and total cell numbers of $CD4^+$, $CD8^+$ and $CD4^+Foxp3^+$ cells from $TCR\alpha\beta^+$ and $CD4^+CD8^+$ cells from $CD45^+$ cells in the thymus are shown in the graphs **(right)**. Data from one representative experiment of three are shown (mean ± SD). ns, not significant; 2-tailed unpaired Student t test analysis.

To analyze the role of Gal-1 in the sensitization phase, Gal-$1^{+/+}$ and Gal-$1^{-/-}$ mice were treated with OXZ in the abdomen **(Figure 5.8a)**. Immune cell populations were analyzed in lymph nodes after 48 h and 5 d. We observed that, after the first challenge with the hapten, Gal-$1^{-/-}$ mice displayed the same total number of $CD4^+$, $CD8^+$ and Treg compared to Gal-$1^{+/+}$ mice **(Figure 5.8b, c)**. Moreover, the neutrophilic infiltration found in the skin after 48 h of the first challenge with OXZ was similar between both genotypes **(Figure 5.8d)**. Overall, these results indicated that Gal-1 mainly controls the inflammatory response induced after the second challenge with OXZ.

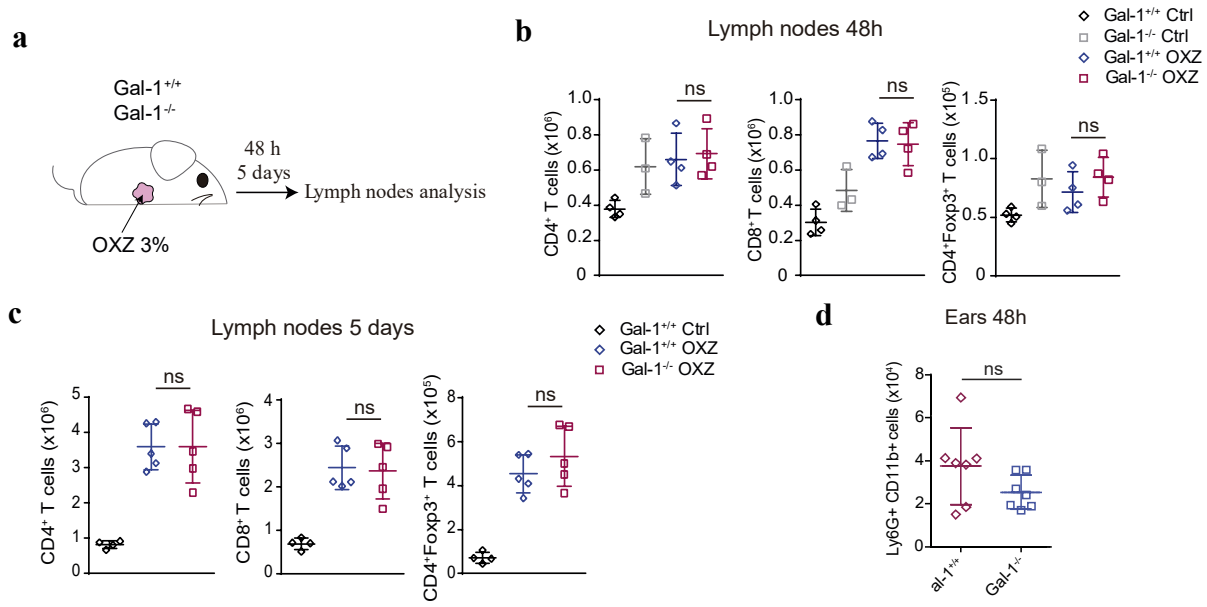

Figure 5.8. Gal-1 does not regulate the sensitization phase of CHS. (a) Gal-$1^{-/-}$ and Gal-$1^{+/+}$ mice were treated with OXZ in the abdomen and sacrificed after 48 h and 5 d. Flow cytometry analysis of total number of $CD4^+$, $CD8^+$ and $CD4^+Foxp3^+$ T cells in lymph nodes at 48 h **(b)** and 5 d **(c)** after OXZ. **(d)** Total numbers of neutrophils ($CD11b^+Ly6G^+$) detected in the skin 48 h after the sensitization phase are shown. Individual data (mean ± SD) from one representative experiment of three were shown. ns, not significant; 1-way ANOVA with the Bonferroni post hoc test **(b, c)**; 2-tailed unpaired Student t test analysis **(d)**.

Besides, our data showed a delay in the migration of DCs from the skin to the lymph nodes at 48 h, in the absence of Gal-1. However, this effect of Gal-1 vanished at 72 h after sensitization phase **(Figure 5.9)**.

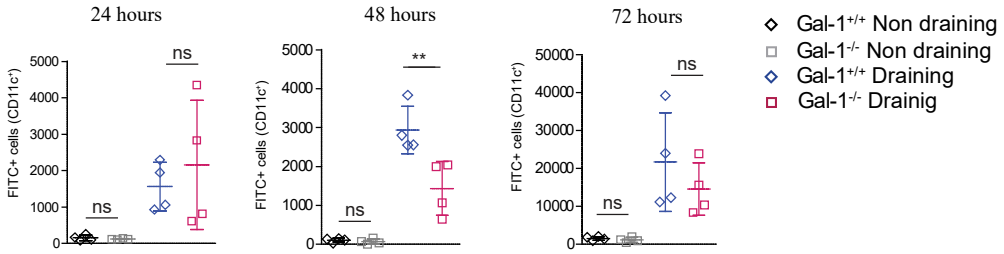

Figure 5.9. Gal-1$^{-/-}$ mice display a delay in DCs migration from the skin to the lymph nodes. Total cell numbers of CD11c^{+}FITC^{+} cells from CD3^{-}MHCII^{+} cells in the lymph nodes are shown in the graphs. Data from one representative experiment of three are shown (mean ± SD). ns, not significant; **P < 0.01 1-way ANOVA with the Bonferroni post hoc test.

IL-17^{+}γδ T cells are relevant mediators of skin inflammatory diseases such as ACD (39) and psoriasis (162). To address the role of Gal-1 in γδ T cells in the CHS model, we studied this population using the gating strategy indicated in **Figure 5.10**.

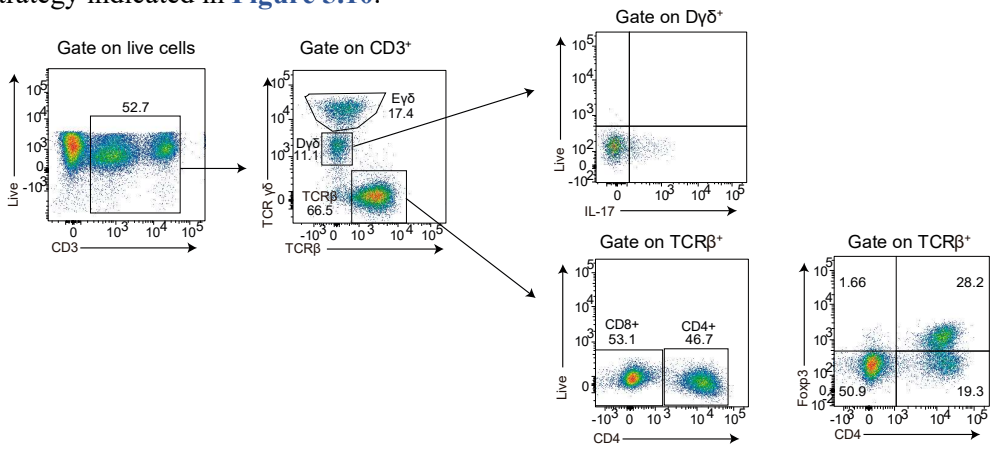

Figure 5.10. Diagram of the gating strategy used to analyze the different subsets of T cells detected in the skin diagram. Dermal (Dγδ) and epidermal (Eγδ) γδ T cells.

The number of dermal γδ T cells but not dendritic epidermal γδ T cells increased by day 7 after the elicitation phase in Gal-1$^{-/-}$ in comparison to Gal-1$^{+/+}$ mice **(Figure 5.11)**. Interestingly, kinetic analyses of lymphocyte infiltration of OXZ-treated ears demonstrated that Gal-1 deficiency increases the total number of effector CD8^{+} **(Figure 5.11)** and IL-17^{+}γδ **(Figure 5.12)** T cells at day 7 after treatment.

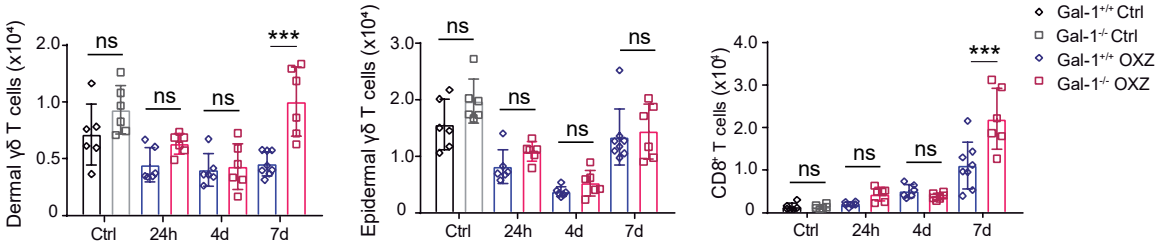

Figure 5.11. Increased effector T cells recruitment in the absence of Gal-1 expression. (a) Total cell numbers of dermal γδ^{+}, epidermal γδ^{+} and CD8^{+} T cells detected in the skin of OXZ-treated or untreated (Ctrl) Gal-1$^{-/-}$ and Gal-1$^{+/+}$ mice are shown in the graphs. Individual data (mean ± SD) from one representative experiment of three were shown. ns, not significant; ***P < 0.001, 2-way ANOVA with the Bonferroni post hoc test.

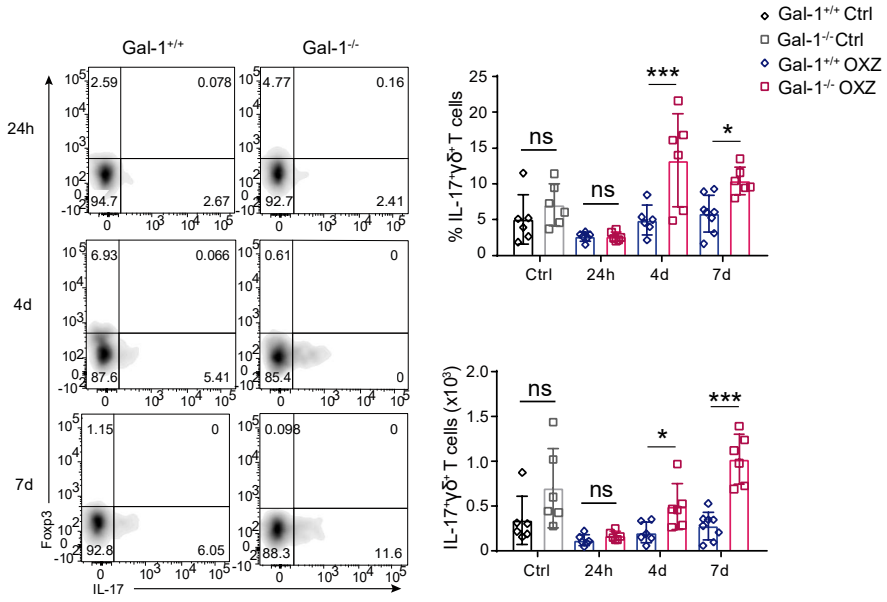

Figure 5.12. Increased IL-17$^+$ γδ T cells recruitment in the absence of Gal-1 expression. Density plots **(left)**, frequencies and total numbers of IL-17$^+$ γδ T cells **(right)** quantified in the ears. Individual data (mean ± SD) from one representative experiment of three were shown. ns, not significant; *P < 0.05 and ***P < 0.001, 2-way ANOVA with the Bonferroni post hoc test.

In contrast, Gal-1$^{-/-}$ mice are not significantly different from Gal-1$^{+/+}$ in terms of the numbers of CD4$^+$ and CD4$^+$Foxp3$^+$ cells over time **(Figure 5.13)**. These data indicate that Gal-1$^{-/-}$ mice develop a sustained inflammation at day 7 after OXZ compared to Gal-1$^{+/+}$ mice, likely due to an increase of CD8$^+$ and IL-17-secreting γδ T cells.

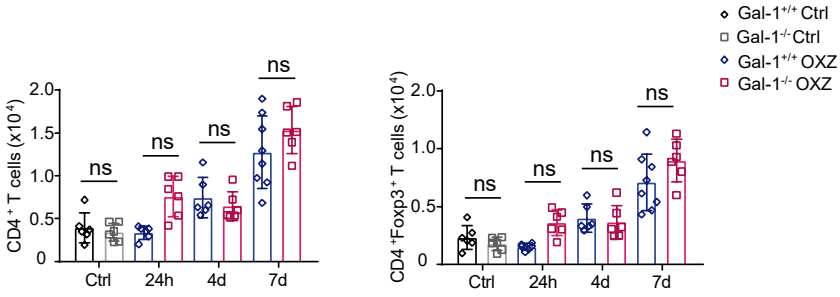

Figure 5.13. Gal-1$^{+/+}$ and Gal-1$^{-/-}$ mice did not show differences in the number of CD4$^+$ and CD4$^+$Foxp3$^+$ cells over time. Total cell numbers of CD4$^+$ T cells and CD4$^+$Foxp3$^+$ T cells in the skin of Gal-1$^{-/-}$ and Gal-1$^{+/+}$ mice are shown. Individual data (mean ± SD) from one representative experiment of three were shown. ns, not significant; 2-way ANOVA with the Bonferroni post hoc test.

5.1.3. Gal-1$^{-/-}$ and Gal-1$^{+/+}$ T cells display similar migratory ability to the inflamed skin

Gal-1 can be found in circulation as soluble protein, as well as it can be expressed by endothelial cells (164, 165). To study whether the Gal-1-mediated effect in CHS is related to the adhesion and migration of T cells to OXZ-treated skin, we induced CHS in the presence of a blocking anti-ICAM-1 antibody (166). We found that Gal-1$^{-/-}$ mice still displayed increased inflammation compared to Gal-1$^{+/+}$ mice, ruling out the involvement of ICAM-1 in the anti-inflammatory effect of Gal-1 in the CHS response induced by OXZ **(Figure 5.14)**.

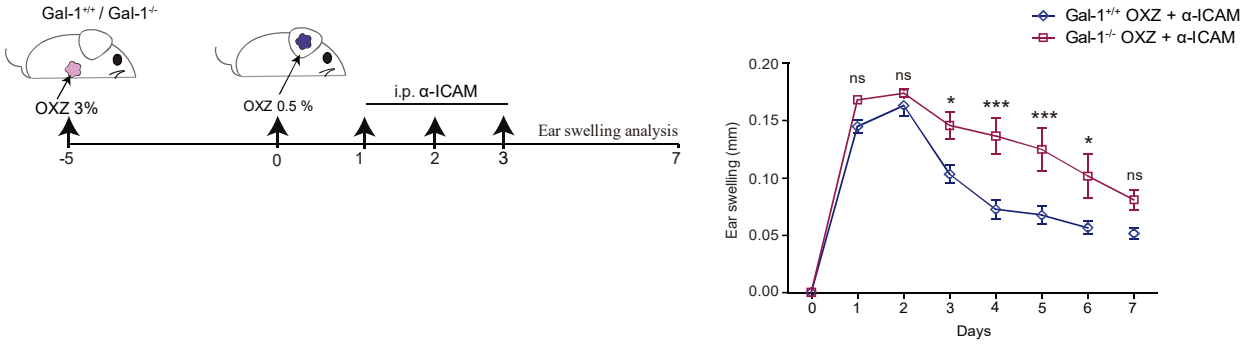

Figure 5.14. ICAM-1 does not participate in the anti-inflammatory effect of Gal-1 in the CHS response. Ear thickness of Gal-1$^{+/+}$ and Gal-1$^{-/-}$ treated with OXZ and anti-ICAM-1 antibody. Individual data (mean ± SD) from one representative experiment of three were shown. ns, not significant; *P < 0.05 and ***P < 0.001, 2-way ANOVA with the Bonferroni post hoc test.

To further assess the role of soluble and endothelial Gal-1 in the migration of immune cells to inflamed skin and lymph nodes, WT cells from dLNs of OXZ-treated mice were intravenously injected in Rag1$^{-/-}$Gal-1$^{-/-}$ mice or Rag1$^{-/-}$Gal-1$^{+/+}$ **(Figure 5.15a)**. Flow cytometry data revealed that Gal-1$^{+/+}$ T cells (CD4^{+} and CD8^{+}) similarly migrate to the ears of Rag1$^{-/-}$Gal-1$^{-/-}$ and Rag1$^{-/-}$Gal-1$^{+/+}$ recipient mice **(Figure 5.15b)**. Furthermore, circulating or endothelial Gal-1 expression does not play a relevant role in CD4^{+} and CD8^{+} T cell entrance to or exit from to the lymph nodes **(Figure 5.15b)**.

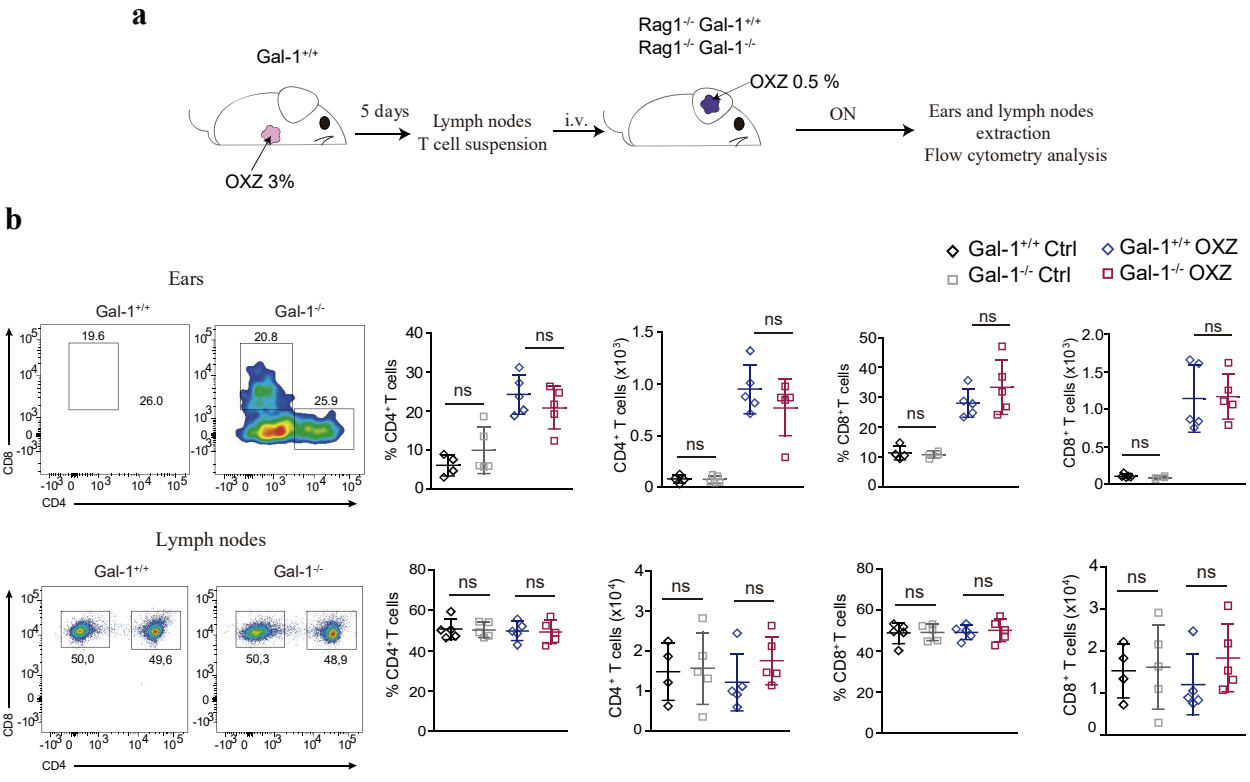

Figure 5.15. Gal-1 expression does not control differential migration of T cells to inflamed skin. **(a)** Rag1$^{-/-}$ Gal-1$^{+/+}$ and Rag1$^{-/-}$ Gal-1$^{-/-}$ mice were sensitized by applying OXZ (0.5 %) and were injected with Gal-1$^{+/+}$ T cell suspension isolated from OXZ-treated mice. **(b)** Representative plots of CD4^{+} and CD8^{+} T cells populations of ears **(upper)** and lymph nodes **(bottom)** from Rag1$^{-/-}$ Gal-1$^{+/+}$ and Rag1$^{-/-}$ Gal-1$^{-/-}$ recipient mice. Individual values of frequencies and total cell numbers are shown in the graphs. Individual data (mean ± SD) from one representative experiment of three were shown. ns, not significant; 1-way ANOVA with the Bonferroni post hoc test.

5.1.4. Endogenous expression of Gal-1 in T cell compartment regulates the development of CHS

To ascertain whether deletion of Gal-1 in bone marrow-derived lymphoid and myeloid cells could recapitulate the enhanced and sustained inflammation observed in the CHS model, we analyzed the inflammation in CD45.1^{+}Gal-1$^{+/+}$ mice lethally irradiated and bone marrow-transferred (BMT) with Gal-1$^{+/+}$ or Gal-1$^{-/-}$ cells **(Figure 5.16a)**. We found that the absence of Gal-1 in hematopoietic cells recapitulates the sustained inflammation observed in Gal-1 full-deficient mice **(Figure 5.16b)**.

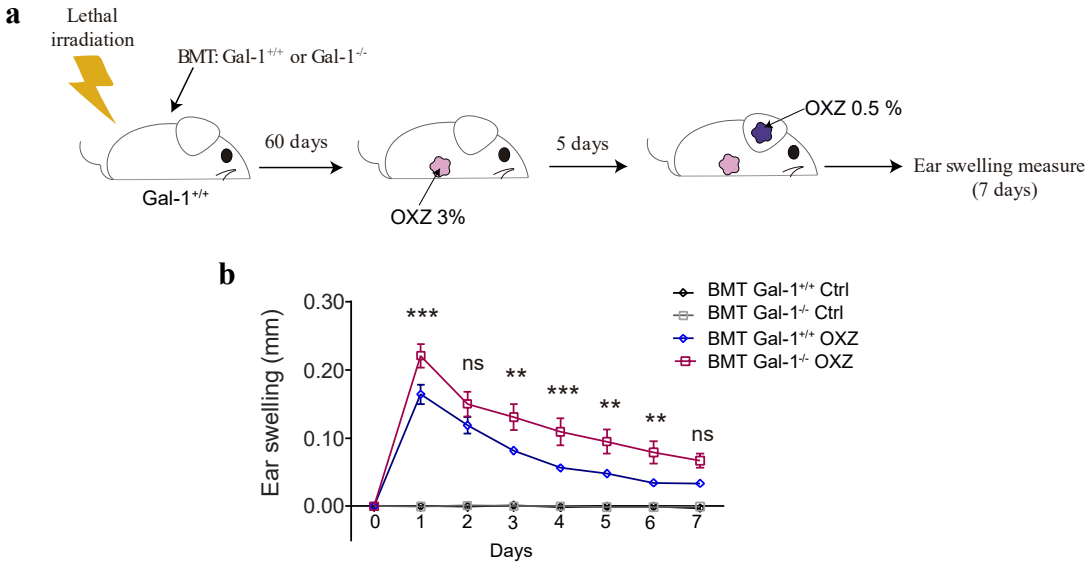

Figure 5.16. The absence of Gal-1 in hematopoietic cells in BMT-mice recapitulates the sustained inflammation observed in CHS model (a) WT CD45.1 recipient mice were lethally irradiated and bone marrow transferred with Gal-1$^{+/+}$ and Gal-1$^{-/-}$ cells. CHS response was assessed in chimeric mice after 60 d. **(b)** Results are expressed as the mean ear swelling ± SD at different time points after challenge, and are representative of three independent experiments. ns, not significant; **P < 0.01 and ***P < 0.001, 2-way ANOVA with the Bonferroni post hoc test.

To directly assess whether endogenous Gal-1 expression in T cells mediates CHS, we transferred CD45.2^{+} T lymphocytes from OXZ-treated-Gal-1$^{-/-}$ and -Gal-1$^{+/+}$ mice into CD45.1^{+}Gal-1$^{+/+}$ recipient mice **(Figure 5.17a)**.

After OXZ challenge, mice receiving Gal-1$^{-/-}$ T cells displayed more sustained inflammation than those transferred with Gal-1$^{+/+}$ T cells, indicating that endogenous expression of Gal-1 in T cells, but not in the myeloid compartment, is sufficient to control CHS **(Figure 5.17b)**. Furthermore, flow cytometry analysis demonstrated higher numbers of CD45.2^{+}Gal-1$^{-/-}$ CD4^{+}, CD8^{+} and Treg cells in lymph nodes **(Figure 5.17c)**. These results confirm that the absence of endogenous Gal-1 increases the reactivation of T lymphocytes after the second challenge with OXZ, which promotes the development of the disease.

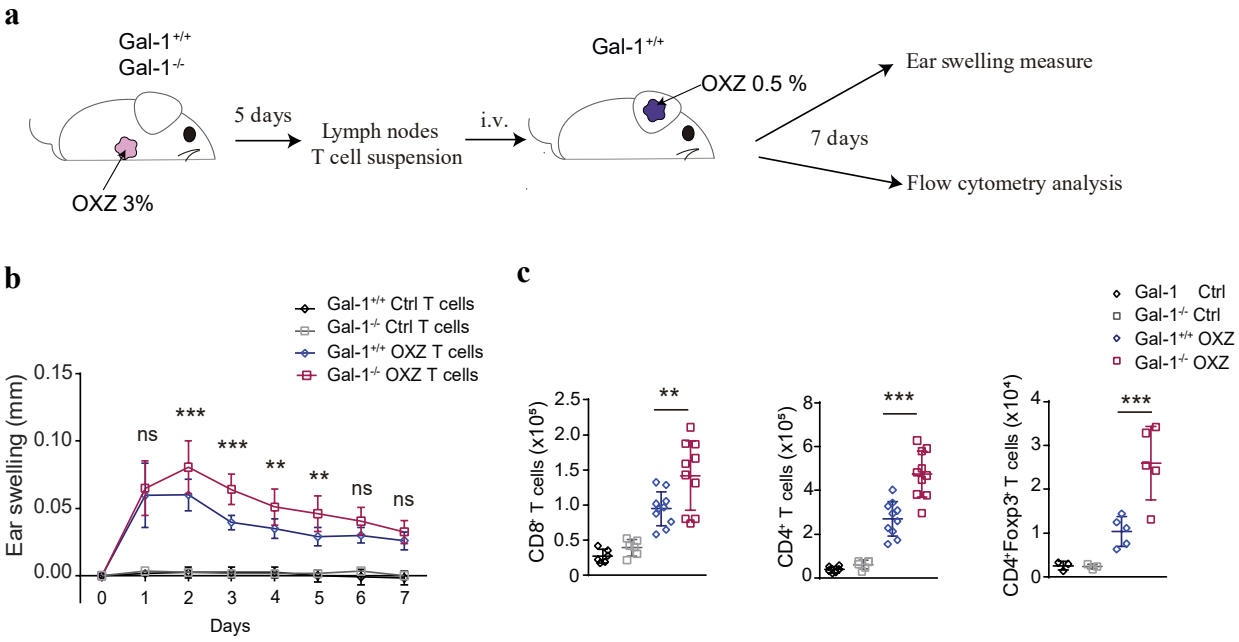

Figure 5.17. Gal-1 expression plays a major role in T cell compartment in CHS. (a) WT recipient mice injected with T cells from OXZ-challenged Gal-1$^{-/-}$ and Gal-1$^{+/+}$ mice were treated with OXZ. **(b)** Ear thickness is represented as the mean ear swelling ± SD at different time points after challenge. **(c)** Total cell numbers of CD8^{+}, CD4^{+} and CD4^{+}Foxp3^{+} lymph nodes are shown in the graphs. Individual data (mean ± SD) from one representative experiment of three is shown. ns, not significant; **P < 0.01 and ***P < 0.001, 1-way ANOVA **(c)** or 2-way ANOVA **(b)** with the Bonferroni post hoc test.

5.1.5. CD4^{+}Foxp3^{+} T cells are functional in the absence of Gal-1 expression

To address whether the expression of Gal-1 in CD4^{+}Foxp3^{+} Treg cells accounts for the phenotype observed in Gal-1$^{-/-}$ mice, adoptive transfer of CD4^{+}Foxp3^{+} or CD4^{+}Foxp3^{-} T lymphocytes isolated from OXZ-treated-Gal-1$^{-/-}$ and -Gal-1$^{+/+}$ mice into Gal-1$^{+/+}$ recipient mice was conducted **(Figure 5.18a)**. After OXZ challenge, mice receiving CD4^{+}Foxp3^{+} T cells from both genotypes showed similar reduced inflammatory response, suggesting that the regulatory capacity of CD4^{+} Foxp3^{+} cells is not affected by deletion of Gal-1 in CHS model. Besides, mice receiving effector CD4^{+}Foxp3^{-}Gal-1$^{-/-}$ and Gal-1$^{+/+}$ T cells displayed a similar increased inflammatory response **(Figure 5.18b)**.

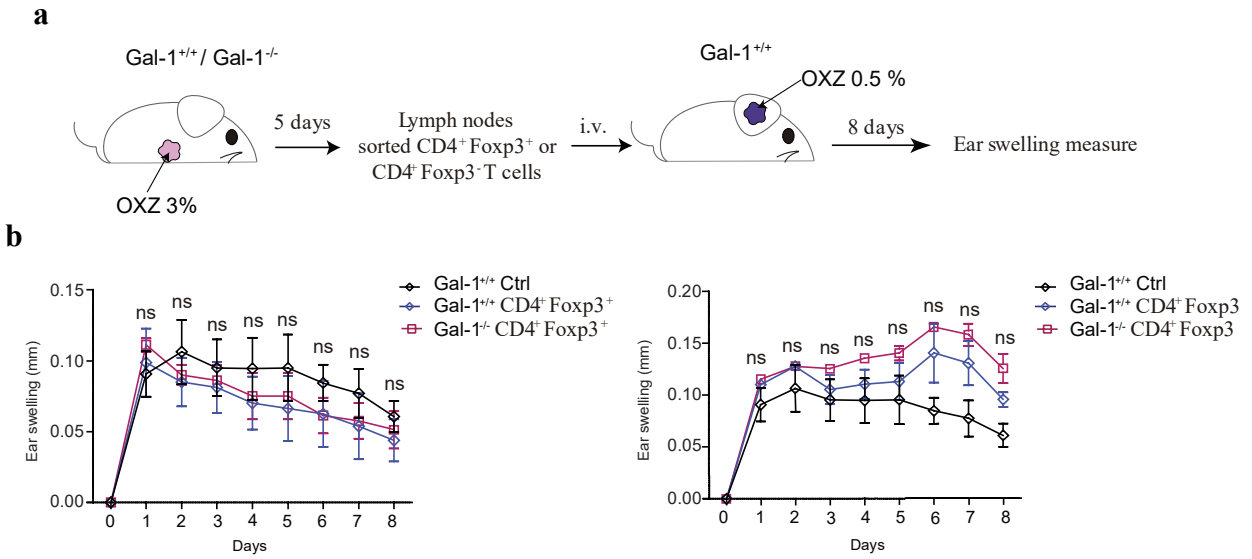

Figure 5.18. CD4⁺ Foxp3⁺ and CD4⁺ Foxp3⁻ T cells are not affected by deletion of Gal-1 in CHS model **(a)** WT recipient mice transferred with sorted CD4⁺Foxp3⁺ or CD4⁺Foxp3⁻ T cells from OXZ-challenged Gal-1$^{-/-}$ and Gal-1$^{+/+}$ mice, were treated with OXZ. **(b)** Ear thickness increase is shown. Individual data (mean ± SD) from one representative experiment of three is shown. ns, not significant; 2-way ANOVA with the Bonferroni post hoc test.

We evaluate the ability of CD4⁺Foxp3⁺ T cells derived from OXZ-treated Gal-1$^{-/-}$ and Gal-1$^{+/+}$ mice to control CD8⁺ T cell proliferation *ex vivo*. Inhibition of CD8⁺ T cell proliferation was similarly mediated by CD4⁺Foxp3⁺ T cells from Gal-1$^{-/-}$ and Gal-1$^{+/+}$ mice **(Figure 5.19a)**. In addition, the absence of Gal-1 does not seem to modify the expression of GITR in CD4⁺Foxp3⁺ T cells **(Figure 5.19b)**. Overall, these results indicate that deletion of Gal-1 in CD4⁺ T cells, either effector or regulatory cell, does not affect CHS response induced by OXZ.

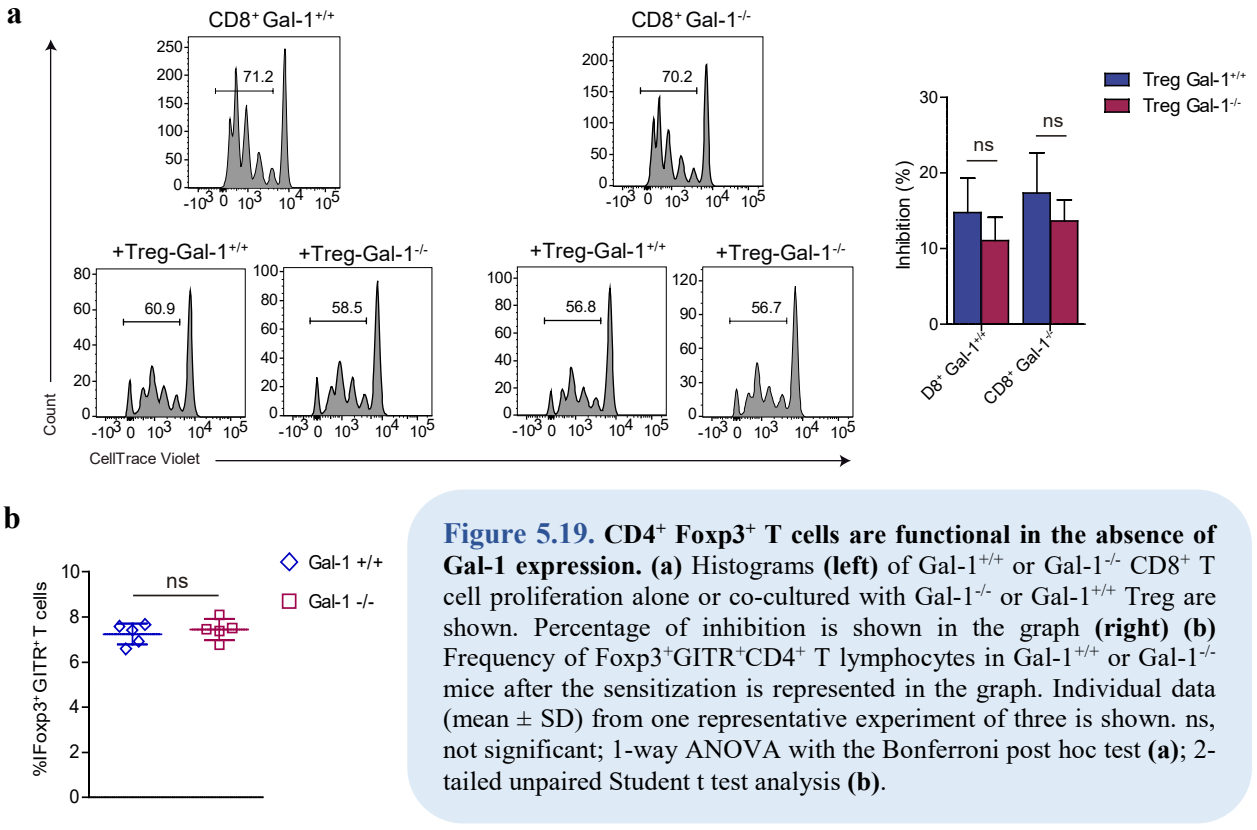

Figure 5.19. CD4⁺ Foxp3⁺ T cells are functional in the absence of Gal-1 expression. (a) Histograms **(left)** of Gal-1$^{+/+}$ or Gal-1$^{-/-}$ CD8⁺ T cell proliferation alone or co-cultured with Gal-1$^{-/-}$ or Gal-1$^{+/+}$ Treg are shown. Percentage of inhibition is shown in the graph **(right) (b)** Frequency of Foxp3⁺GITR⁺CD4⁺ T lymphocytes in Gal-1$^{+/+}$ or Gal-1$^{-/-}$ mice after the sensitization is represented in the graph. Individual data (mean ± SD) from one representative experiment of three is shown. ns, not significant; 1-way ANOVA with the Bonferroni post hoc test **(a)**; 2-tailed unpaired Student t test analysis **(b)**.

5.1.6. Gal-1-mediated control of CHS response is restricted to CD8$^+$ T cell compartment

To ascertain the relevance of Gal-1 in CD4$^+$ T cells versus CD8$^+$ T lymphocytes, *in vivo* CD4$^+$ T cell depletion was performed in Gal-1$^{-/-}$ and Gal-1$^{+/+}$ mice **(Figure 5.20a)**. The differential inflammatory response detected between both genotypes is maintained in the absence of CD4$^+$ T cells **(Figure 5.20b)**, indicating a major role for Gal-1 in CD8$^+$ T cell compartment. Besides, total number of CD8$^+$ T cells and their proliferation response in skin tissue **(Figure 5.20c)**, and lymph node **(Figure 5.20d)** were similar in Gal-1$^{-/-}$ and Gal-1$^{+/+}$ mice, regarding the presence or absence of CD4$^+$ T cells.

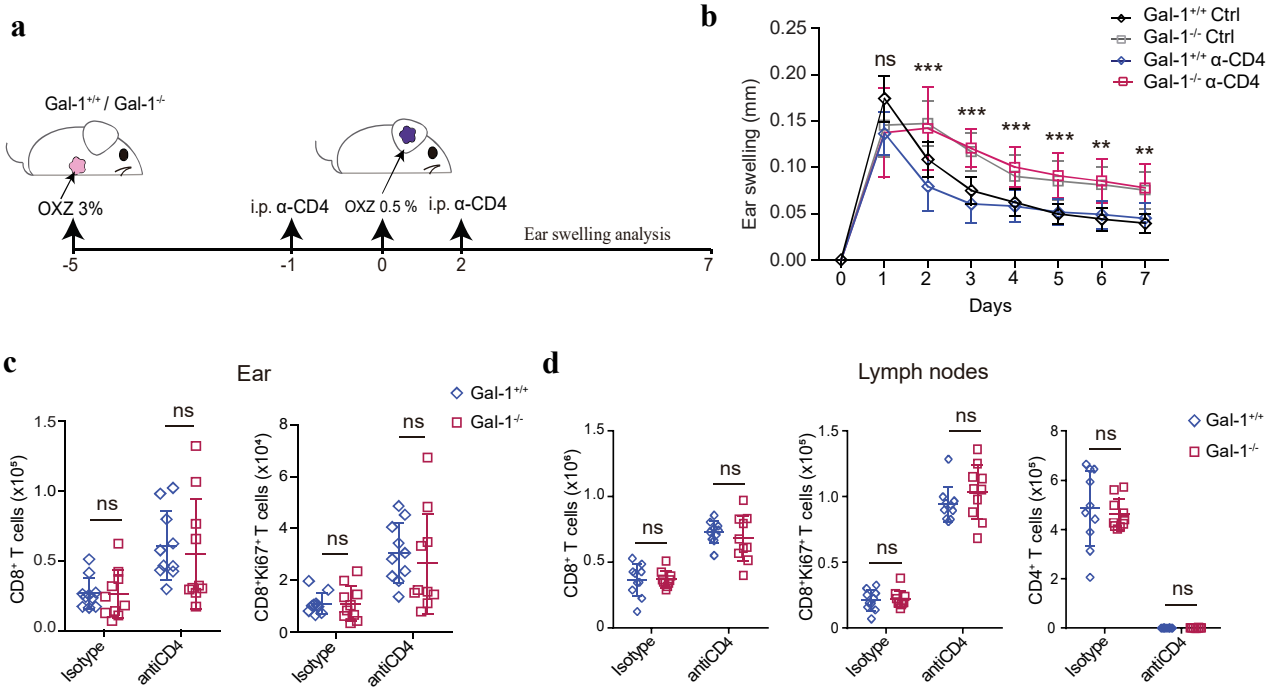

Figure 5.20. The differential CHS response detected between both genotypes is maintained in the absence of CD4$^+$ T cells. (a) *In vivo* anti-CD4 treatment. **(b)** Ear thickness was assessed. CD8$^+$ and CD8$^+$Ki67$^+$ T cells in ears **(c)** and lymph nodes **(d)** were assessed. Data (mean ± SD) from one experiment out of three. ns, not significant; **P < 0.01 and ***P < 0.001, 1-way ANOVA **(c, d)** or 2-way ANOVA **(b)** with the Bonferroni post hoc test.

The transfer of CD8$^+$ T cells derived from OXZ-treated Gal-1$^{-/-}$ mice was sufficient to recapitulate the phenotype of full Gal-1$^{-/-}$ mice **(Figure 5.21)**.

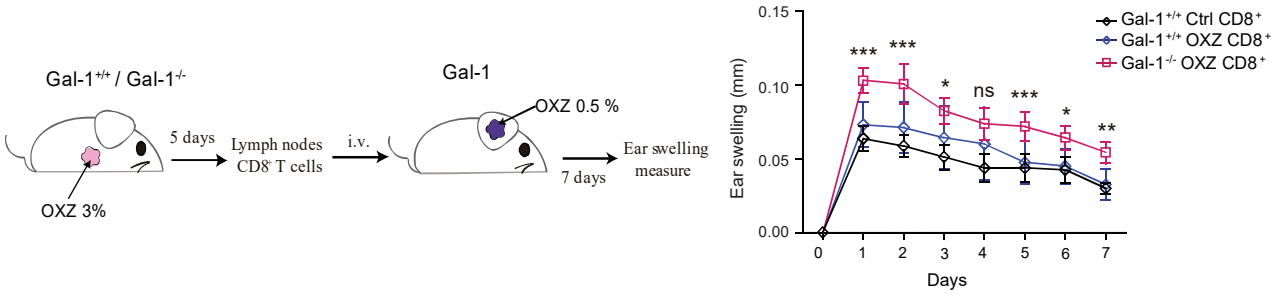

Figure 5.21. Major role for Gal-1 in CD8$^+$ T cell compartment. Mice transferred with CD8$^+$ T cells from OXZ-challenged Gal-1$^{-/-}$ and Gal-1$^{+/+}$ mice received OXZ **(left)**. Ear swelling is shown **(right)**. Data (mean ± SD) from one experiment out of three. ns, not significant; *P < 0.05, **P < 0.01 and ***P < 0.001, 2-way ANOVA with the Bonferroni post hoc test.

Interestingly, increased fraction of $CD44^+CD62L^+CD8^+$ T cells but not $CD44^+CD62L^+CD4^+$ T cells, corresponding to central memory compartment, was detected in Gal-$1^{-/-}$ mice after the sensitization phase **(Figure 5.22)**.

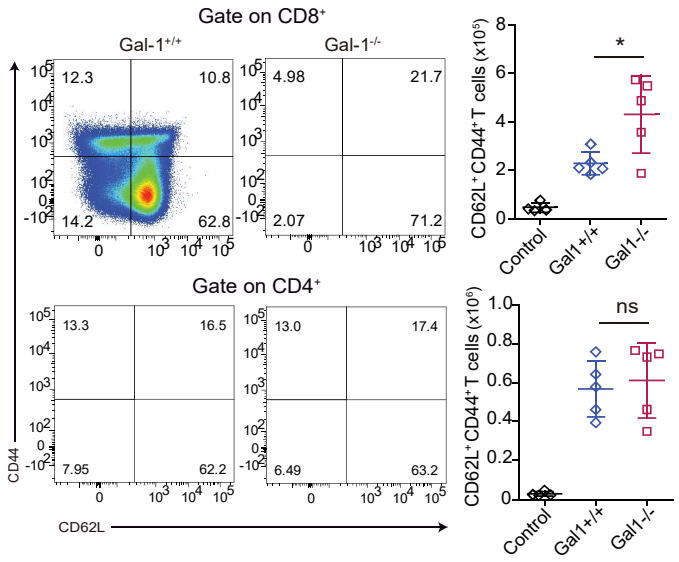

Figure 5.22. Gal-1 deletion increases the development of central memory $CD8^+$ T cells. Representative plots and total number of $CD8^+CD62L^+CD44^+$ and $CD4^+CD62L^+CD44^+$ T cell populations. Data (mean ± SD) from one experiment out of three. ns, not significant; *P < 0.05, 1-way ANOVA with the Bonferroni post hoc test.

Although the expression of T-bet, Granzyme B, EOMES, and PD-1 is similar in activated $CD8^+$ T cells from Gal-$1^{-/-}$ and Gal-$1^{+/+}$ mice **(Figure 5.23a)**, a significant increase of IFNγ is detected in $CD8^+$ T cells deficient for Gal-1 **(Figure 5.23b)**. Moreover, reduced frequencies of IL-10^+ and IL-4^+CD4^+ T cells were detected in Gal-1-deficient mice, as compared to Gal-$1^{+/+}$ mice **(Figure 5.23b)**. These results indicate that Gal-1 deletion increases the development of central memory and IFNγ-secreting effector $CD8^+$ T cells, which induce an exacerbated CHS response.

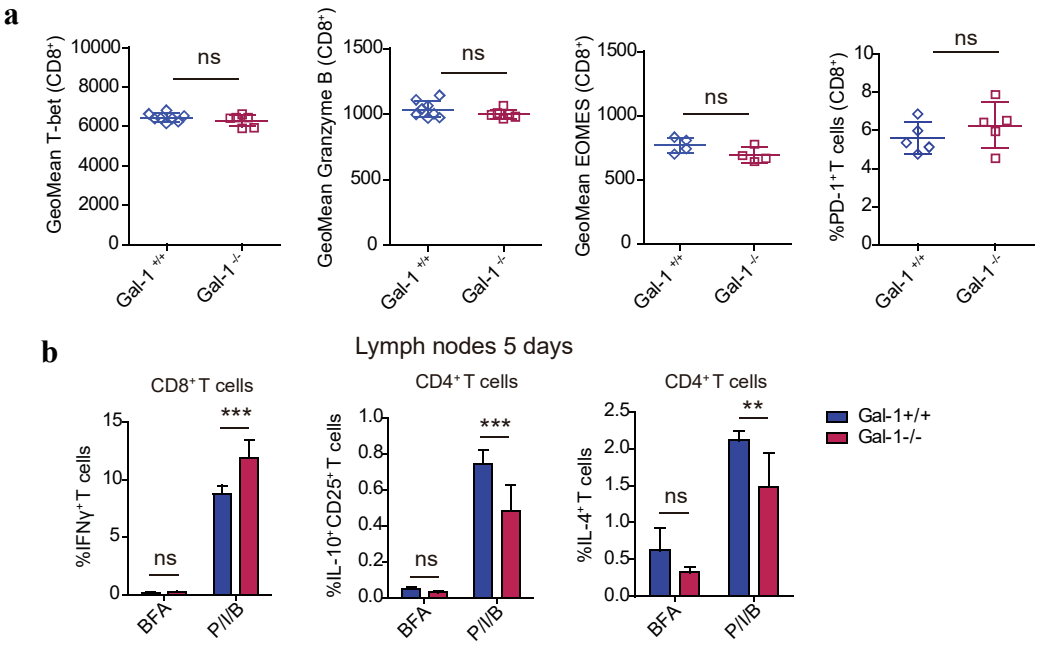

Figure 5.23. Gal-1 deletion increases IFNγ-secreting effector $CD8^+$ T cells. (a) T-bet, Granzyme B, EOMES, and PD-1 expression in $CD8^+$ T cells in lymph nodes. **(b)** Frequency of $CD8^+IFN\gamma^+$, $CD4^+$IL-10^+CD25^+ and $CD4^+$IL-4^+ T cells in lymph nodes. Data (mean ± SD) from one experiment out of three. ns, not significant; *P < 0.05, **P < 0.01 and ***P < 0.001, 2-tailed unpaired Student t test **(a)**, 1-way ANOVA with the Bonferroni post hoc test **(b)**.

5.2. RELEVANT ROLE OF LAT1 IN THE DEVELOPMENT OF PSORIASIS

5.2.1. Expression of essential amino acid transporter LAT1 in psoriasis

To assess the relevance of essential amino acids in psoriasis, we evaluated the levels of L-Leu in WT and immunodeficient mice (Rag1$^{-/-}$), treated or not, with IMQ. The circulating levels of L-Leu in Rag1$^{-/-}$ mice were increased as compared to WT after IMQ application, suggesting that essential amino acids are likely to be actively uptaken by adaptive B or T cells in the IMQ model **(Figure 5.24)**.

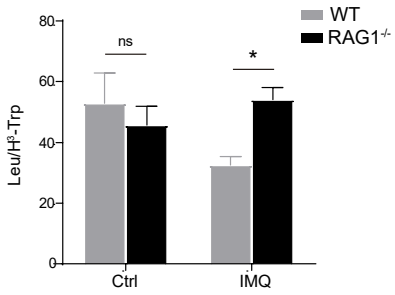

Figure 5.24. Rag1$^{-/-}$ mice showed increased circulating levels of L-Leu compared with WT after skin inflammation. Relative levels of L-Leu detected by using mass spectrometry in sera of healthy (Ctrl) and IMQ-treated WT and Rag1$^{-/-}$ mice (n = 4-6 mice). Bars indicate means ± SEMs. ns, not significant; *P < 0.05, 2-tailed unpaired Student t test.

Normal skin is characterized by LAT2 expression, while LAT1 mainly appears in several different types of skin malignant lesions (167, 168). Our results clearly showed that LAT1 expression is induced in KCs and lymphocytes in human psoriatic skin **(Figure 5.25a, b)**.

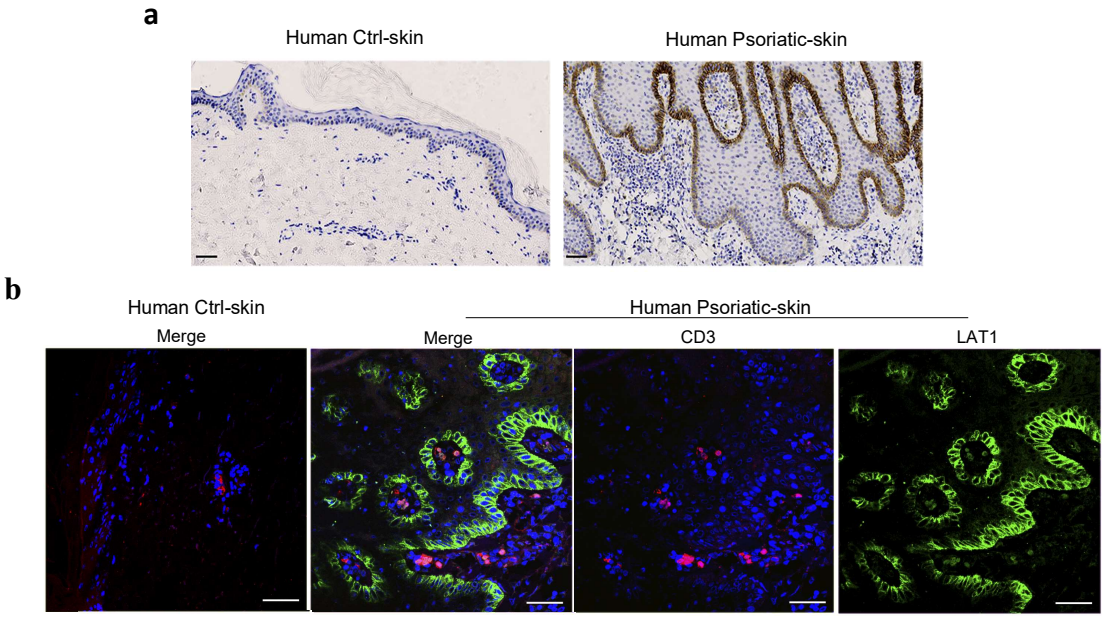

Figure 5.25. Expression of the amino acid transporter LAT1 is induced in patients with psoriasis. (a) LAT1 detection by means of immunohistochemistry in skin biopsy specimens from healthy donors and patients with psoriasis. **(b)** Representative immunofluorescence of LAT1 (green) and CD3 (red) in skin samples from healthy donors and patients with psoriasis. Nuclei were always stained with DAPI (blue). Scale bars = 100 µm. At least three skin biopsy specimens of human origin were simultaneously analyzed.

Similarly, we observed increased LAT1 expression in mouse skin after IMQ application **(Figure 5.26a)**. Further analysis showed that dermal IL-17^{+} cells observed in mice treated with IMQ express LAT1 **(Figure 5.26b)**.

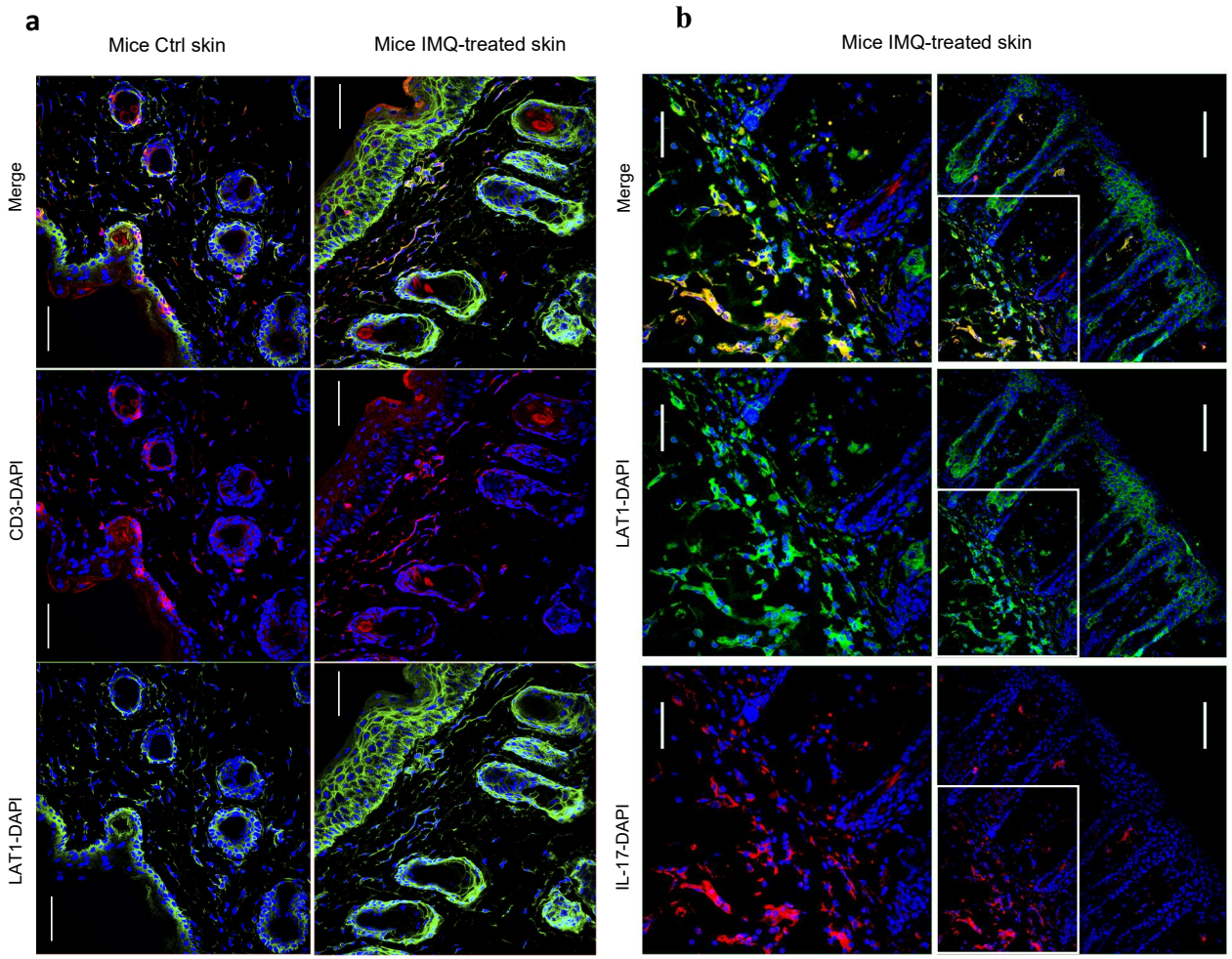

Figure 5.26. Expression of the amino acid transporter LAT1 is induced in the IMQ model. (a) Immunofluorescence of LAT1 (green) and CD3 (red) in skin of WT mice after IMQ and control skin. **(b)** LAT1 (green) expression in dermal cells secreting IL-17 (red) in mice treated with IMQ for 4 d. Zoom areas **(left)** are indicated by the white box **(right)**. Nuclei were stained with DAPI (blue). Scale bars = 100 and 50 µm in zoom area. At least three skin biopsy specimens of mouse origin were analyzed in each experiment.

Considering the high expression of LAT1 in the epidermal layer of psoriatic patients, we examined the effect of deleting LAT1 in KCs. Mice carrying floxed LAT1 alleles ($LAT1^{fl/fl}$) (154) were crossed with mice expressing the Cre recombinase controlled by the keratinocyte-specific Keratin 5 (K5) promoter (K5-CreERT2 mice) (169) and Rosa26-floxed stop tdTomato (Tm) mice (170). This system fluorescently labels the cells that successfully underwent LAT1 deletion. After tamoxifen administration, mice displaying fluorescently red skin from both genotypes, $LAT1^{WT}$ and $LAT1^{\Delta K5}$ were selected for topical treatment with IMQ. Both groups developed psoriasis after IMQ application independent of LAT1 expression in KCs **(Figure 5.27a)**. Increase in epidermal thickness was similar in both genotypes **(Figure 5.27b)**. Neutrophil and macrophage infiltration also increased upon IMQ application, independent of LAT1 expression in epidermal cells **(Figure 5.27c)**.

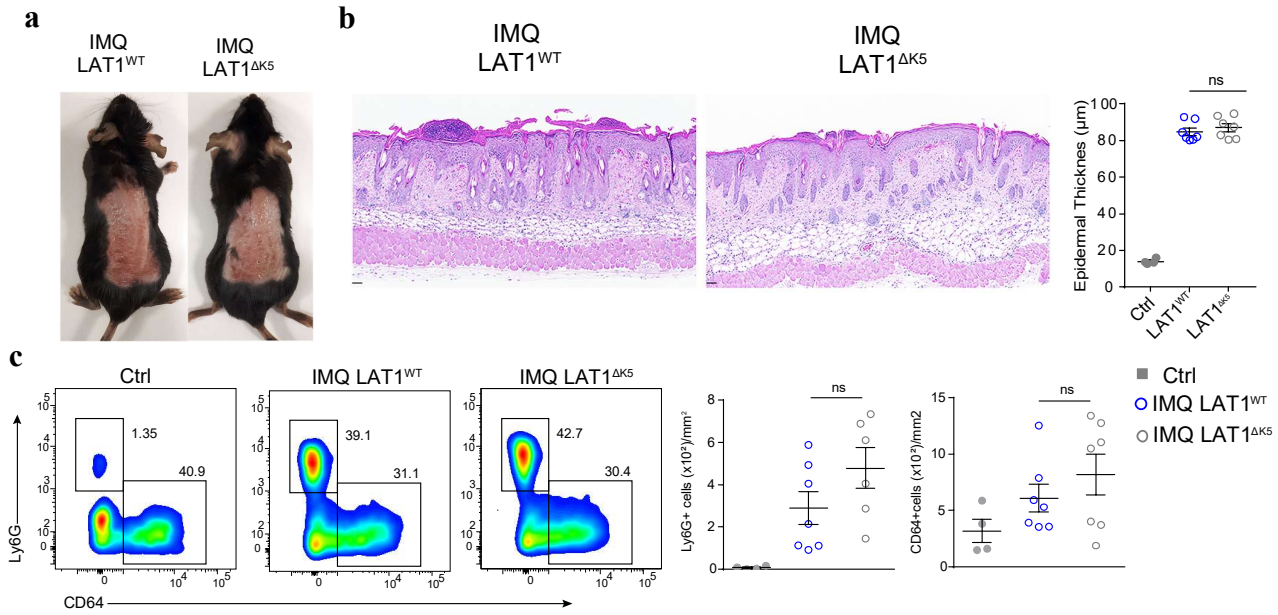

Figure 5.27. Deletion of LAT1 in KCs does not affect psoriasis induced by IMQ. (a) Pictures of LAT1^{WT} and LAT1$^{\Delta k5}$ mice after IMQ application. **(b)** H&E skin sections. Scale bars = 100 µm. Averaged values of epidermal thickness per mice are shown in the graphic at right. **(c)** Density plots of skin neutrophil (Ly6G$^+$; **upper**) and macrophage (CD64$^+$; **right**) populations on CD45$^+$ live cells. Density values of cells are shown in the graphics. A representative experiment of two is shown (n = 4-7 per group). Data are represented as means ± SEMs. ns, not significant; 1-way ANOVA with the Bonferroni post hoc test.

Analysis of Tm$^+$ KCs (CD49f$^+$) confirmed the deletion of LAT1 expression by tamoxifen administration **(Figure 5.28)**.

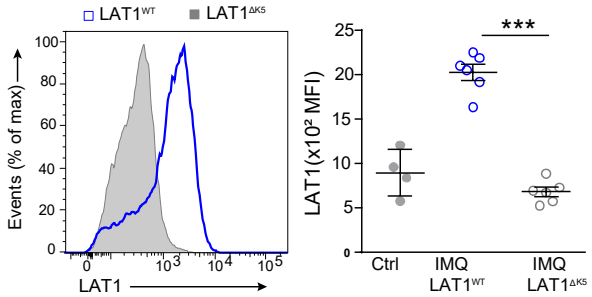

Figure 5.28. LAT1 deletion in Tm$^+$ KCs. Histogram and individual values of LAT1 fluorescence in live KCs. A representative experiment of two is shown (n = 4-7 per group). Data are represented as means ± SEMs. ***P< 0.001; 1-way ANOVA with the Bonferroni post hoc test.

Proliferation markers Ki-67 and BrdU revealed that deletion of LAT1 does not affect KC proliferation **(Figure 5.29)**.

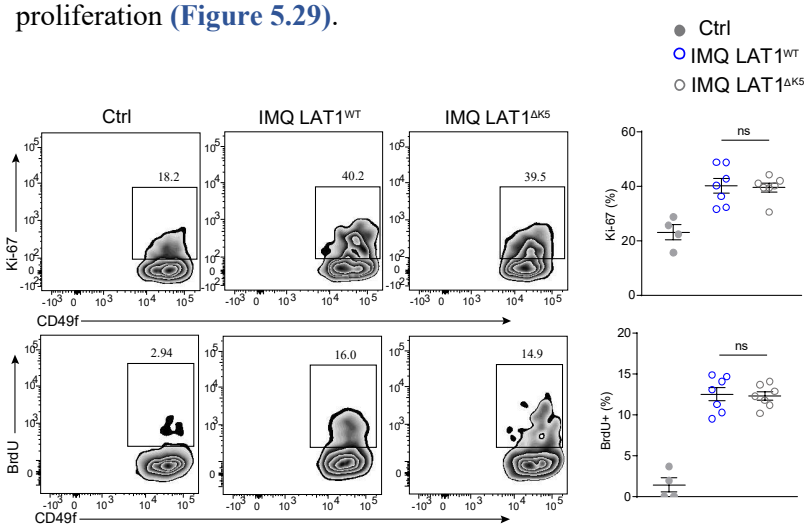

Figure 5.29. KC proliferation is not affected by LAT1 deletion. Density plots **(left)** and individual values of frequencies **(right)** of Ki-67$^+$ **(upper panels)** and BrdU$^+$ **(lower panels)** cells from live KCs. A representative experiment of two is shown (n = 4-7 per group). Data are represented as means ± SEMs. ns, not significant; 1-way ANOVA with the Bonferroni post hoc test.

These results indicate that essential amino acid uptake in these cells does not require LAT1, and could be mediated by other amino acid transporters, such as LAT2 or LAT3, which can also shuttle L-Leu (139). LAT2 is broadly expressed in KCs in normal human skin, and it is increased in psoriatic lesions **(Figure 5.30)**.

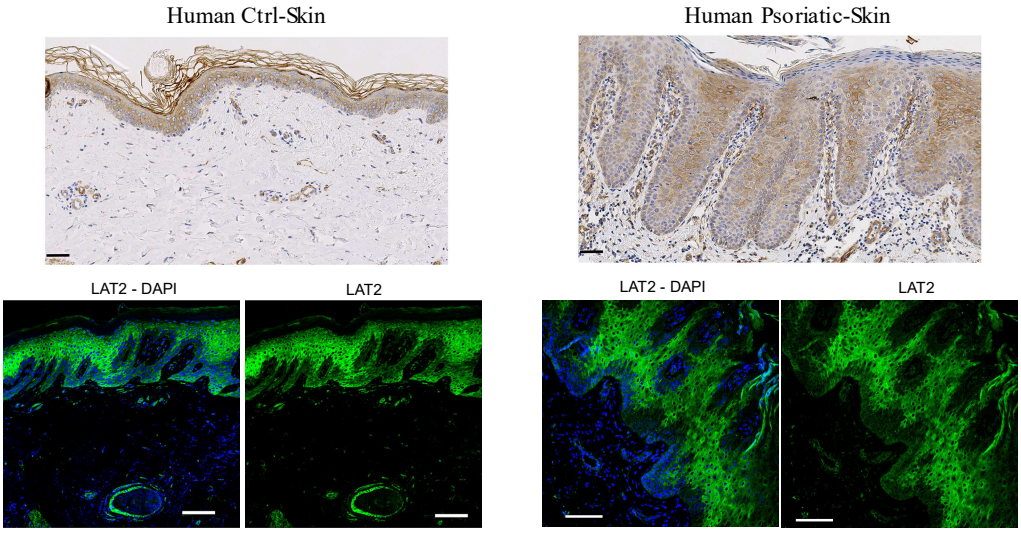

Figure 5.30. LAT2 expression is detected in the epidermal layer in patients with psoriasis. LAT2 detection by means of immunohistochemistry **(upper)** and immunofluorescence **(bottom)** in skin biopsy specimens from healthy donors **(left)** and patients with psoriasis **(right)**. LAT2 are shown in green, and nuclei were stained with DAPI (blue). Scale bars = 100 µm. At least three human skin samples of each condition were simultaneously analyzed in each study.

Epithelial cells such as HaCat and Caco-2 express LAT2 and LAT1, while Hela and lymphoid cells only express LAT1 **(Figure 5.31a)**. Moreover, LAT2 expression in KCs increased in LAT1$^{\Delta K5}$ and LAT1^{WT} mice after IMQ application **(Figure 5.31b)**.

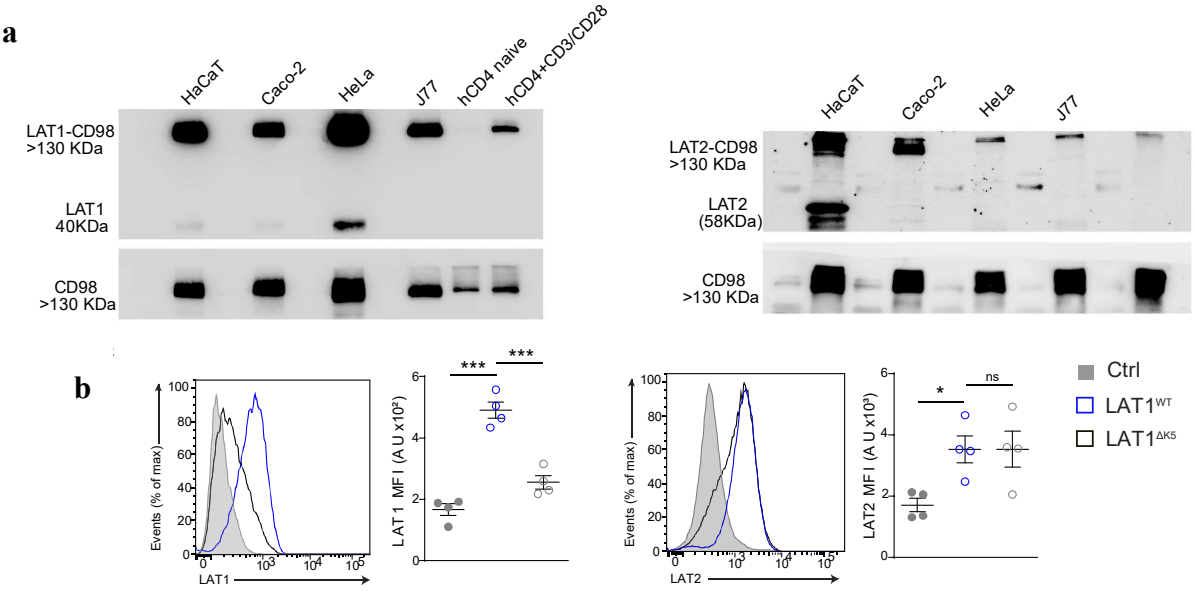

Figure 5.31. LAT2 expression in KCs increased after IMQ. (a) LAT1, LAT2, and CD98 detection by Western blotting (non-reduced) of different types of cell lines and primary human CD4^{+} T cells (naïve and activated). **(b)** Histograms and values of mean fluorescence intensity of LAT1 **(left)** and LAT2 **(right)** expression in LAT1$^{\Delta K5}$ and LAT1^{WT} KCs after IMQ. A representative experiment of at least two individual replicates is shown (n = 4-5). Data are shown as means ± SEM. ns, not significant; *P < 0.05 and ***P < 0.001; 1-way ANOVA with the Bonferroni post hoc test.

Finally, high expression of LAT3 was observed in the basal layer of KCs of normal human skin and psoriatic lesions **(Figure 5.32)**. These data indicate that psoriasis increases the expression of several essential amino acid transporters in KCs to support enhanced proliferation.

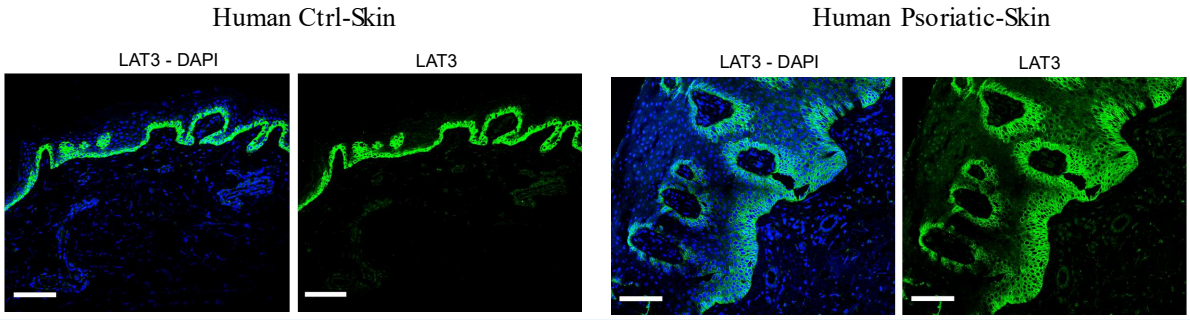

Figure 5.32. LAT3 expression is detected in the epidermal layer in patients with psoriasis. LAT3 detection (green) by means of immunofluorescence in human skin samples. Nuclei were stained with DAPI (blue). Scale bars = 100 µm. At least three human skin samples of each condition were simultaneously analyzed in this study.

Although no differences in KC proliferation were detected between LAT1$^{\Delta K5}$ and LAT1^{WT} mice, we evaluated the inflammatory response induced by IMQ. IL-17^{+} secreting cells detected in the skin of IMQ-treated mice are mainly Vγ4^{+}δ4^{+}T cells and CD4^{+} T cells (171). Importantly, the proliferative response of Vγ4^{+}δ4^{+} T cells **(Figure 5.33a)** and CD4^{+} T cells **(Figure 5.33b)** induced in the dLNs after IMQ application was similar in both mouse groups, independent of LAT1 expression in KCs.

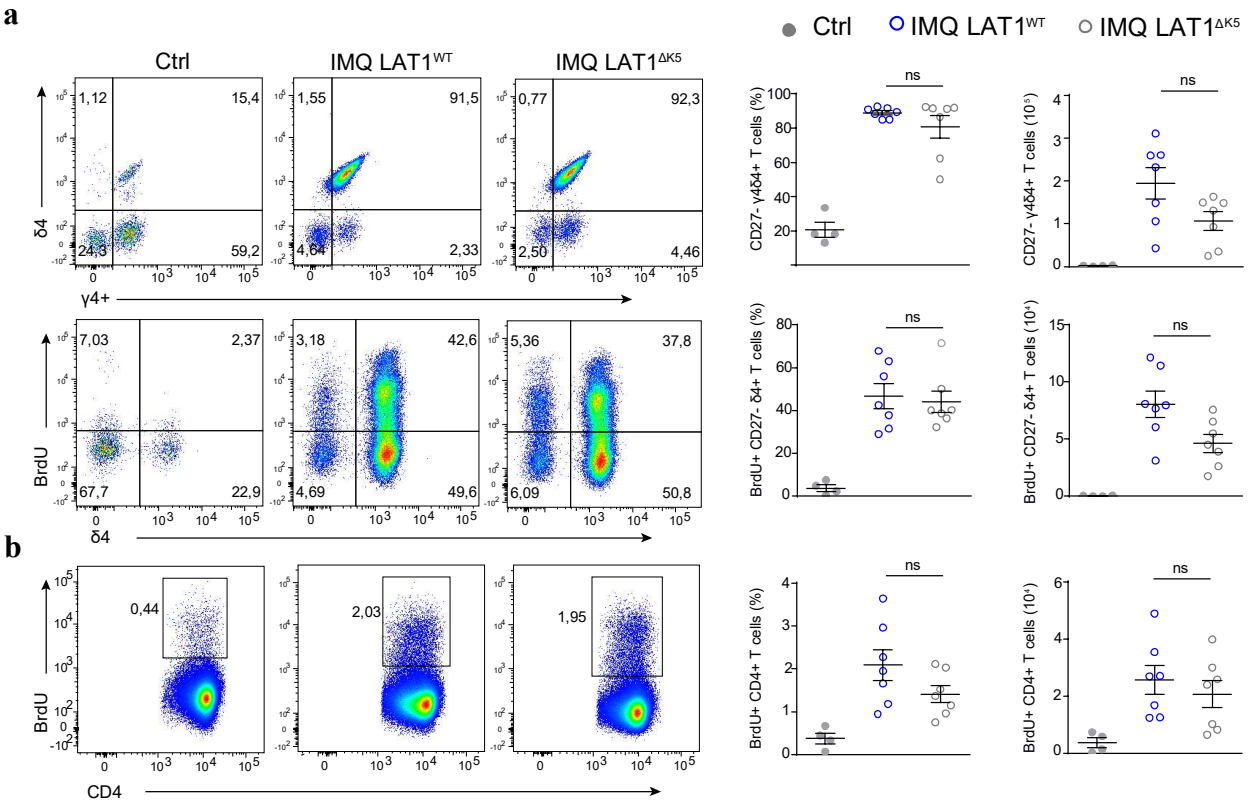

Figure 5.33. The proliferation of Vγ4^{+}δ4^{+} and CD4 T cells after IMQ is independent of LAT1 expression in KCs. (a) Dot plots of Vγ4^{+}δ4^{+} **(upper)** and BrdU^{+}δ4^{+} **(bottom)** cells from live CD27^{-} γδ T cells quantified in the lymph nodes. **(b)** Dot plots of the frequency of BrdU^{+} CD4^{+} T cells in lymph nodes. Individual values of frequencies **(left)** and total cell numbers **(right)** are shown. A representative experiment of two is shown (n = 4-7 per group). Data are represented as means ± SEMs. ns, not significant; 1-way ANOVA with the Bonferroni post hoc test.

5.2.2. LAT1 deletion in innate and adaptive T cells prevents psoriasis

To simultaneously study the function of LAT1 in adaptive and innate lymphocytes, we crossed LAT1$^{fl/fl}$ mice (154) with RORγt-Cre$^{+/-}$ mice (172) and Rosa26-floxed stop tdTomato mice (170). Characterization of LAT1$^{\Delta R\gamma t}$ mice revealed that specific deletion of LAT1 in the skin occurs in cells that had expressed and/or were expressing RORγt, including dermal γδ T cells, skin-resident type 3 innate lymphoid cells, and skin CD4$^+$ T cells **(Figure 5.34a)**. Expression of Cre recombinase was detected in CD27$^-$ γδ T cells within lymph nodes **(Figure 5.34b)**. These are mainly IL-17-secreting cells that also express RORγt (173). Moreover, because RORγt is also expressed during T-cell development at the immature double-positive stage in the thymus (174), deletion of LAT1 can occur also in most TCRαβ T cell subsets **(Figure 5.34b)**.

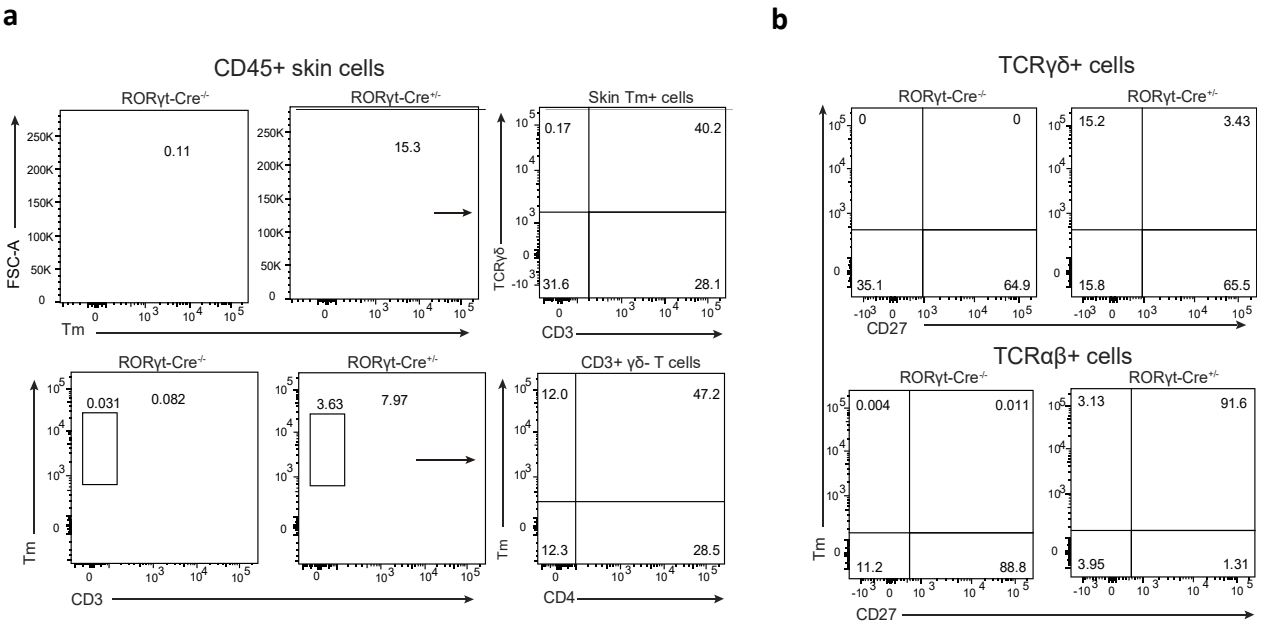

Figure 5.34. Characterization of immune cells with deletion of LAT1 under the control of RORγt expression. (a) Skin Tm$^+$ cells of RORγt-Cre$^{-/-}$ and RORγt-Cre$^{+/-}$ mice were identified as CD3^{low} γδTCR^{low} cells, CD4$^+$ T cells, and CD3$^-$RORγt$^+$ innate lymphoid cells. **(b)** Tm expression observed in CD27$^-$ γδ T cells **(upper)** and CD27$^+$ αβ T cells **(bottom)** from RORγt-Cre$^{+/-}$ mice. A representative experiment of two is shown (n = 3-4).

Indeed, *in vitro*-activated CD4$^+$ T cells from LAT1$^{\Delta R\gamma t}$ mice did not express LAT1 **(Figure 5.35a)**. Also, these cells were smaller and less complex **(Figure 5.35b)** and displayed defective amino acid uptake **(Figure 5.35c)**.

To ascertain whether deletion of LAT1 in RORγt$^+$ cells could affect skin lymphocyte populations at steady state, we analyzed the distribution of different skin CD3$^+$ T-cell subsets. No significant differences in the frequency or absolute numbers of dermal CD4$^+$ and γδ T cells, which were mostly Tm$^+$, were detected in LAT1$^{\Delta R\gamma t}$ and LAT1^{WT} mice at steady state **(Figure 5.36)**. Epidermal γδ T cells (DETC), which were identified by greater TCR expression, did not express RORγt or Tm and were otherwise unaffected by LAT1 deletion in the other subsets **(Figure 5.36)**.

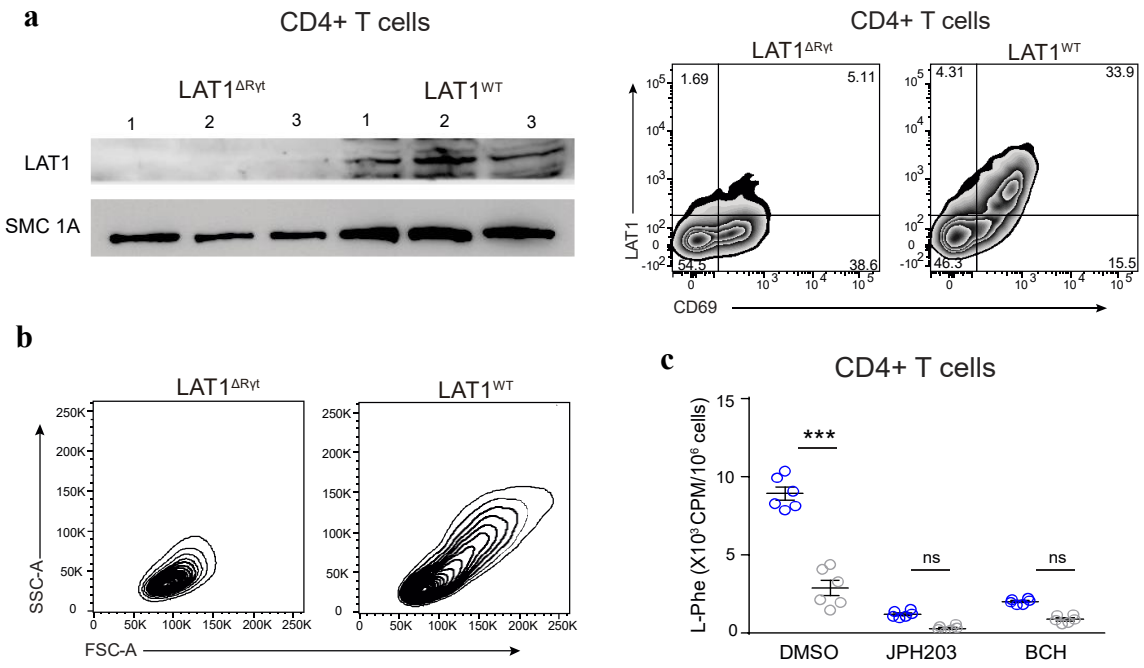

Figure 5.35. Characterization of activated CD4⁺ T cells from LAT1$^{\Delta R\gamma t}$ mice. (a) LAT1 expression was assessed by Western blotting **(left)** and flow cytometry **(right)** in activated CD4⁺ T cells from LAT1^{WT} and LAT1$^{\Delta R\gamma t}$ mice. **(b)** Size and complexity of cells were evaluated by flow cytometry. **(c)** L-Phe uptake was assessed in activated CD4⁺ T cells from LAT1^{WT} and LAT1$^{\Delta R\gamma t}$ mice. A representative experiment of two is shown n = 3-4 **(a, b)** or n = 6 **(c)**. Data are shown as means ± SEMs. ns, not significant; ***P < 0.001; 2-way ANOVA with the Bonferroni post hoc test **(c)**.

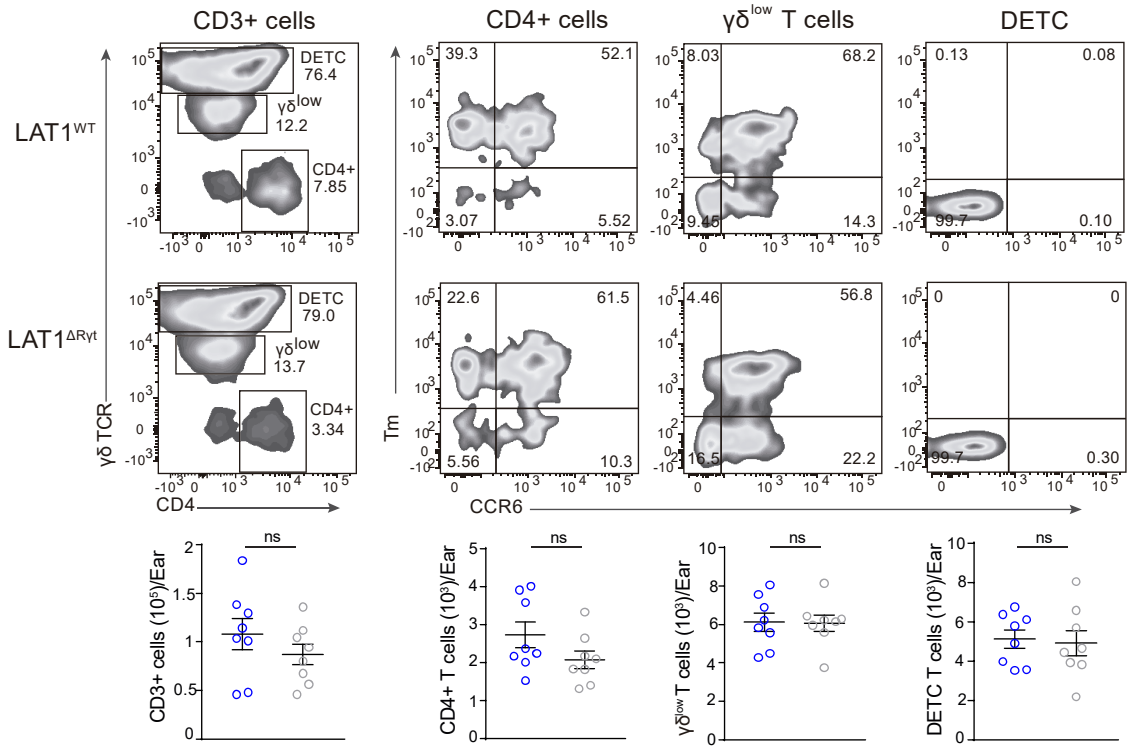

Figure 5.36. Analysis of skin lymphocyte populations at steady state in LAT1$^{\Delta R\gamma t}$ mice. Expression of Tm and CCR6 in skin CD4⁺ T cells, dermal γδ T cells (γδ^{low}), and epidermal γδ T cells (DETC) in LAT1^{WT} and LAT1$^{\Delta R\gamma t}$ mice are shown **(upper)**. Absolute numbers are shown **(bottom)**. A pool of 2 independent experiments is shown (n = 4). Data are shown as means ± SEMs. ns, not significant; 2-tailed unpaired Student t test.

Compared with LAT1^{WT} mice, induction of psoriasis in LAT1$^{\Delta R\gamma t}$ mice was clearly reduced, with a smaller area affected and an almost complete absence of redness and squamous appearance **(Figure 5.37a)**. Protection in LAT1$^{\Delta R\gamma t}$ mice was confirmed by analyzing KC proliferation by means of H&E and Ki-67 staining of skin sections, which was markedly reduced compared with LAT1^{WT} mice **(Figure 5.37b)**.

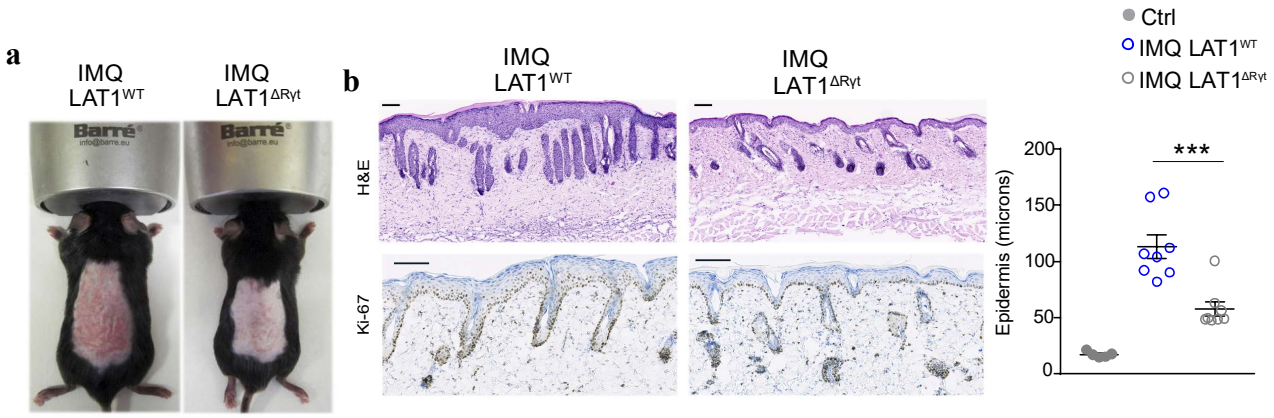

Figure 5.37. Deletion of LAT1 in innate and adaptive T cells prevents IMQ-induced psoriasis. (a) Representative pictures of LAT1^{WT} and LAT1$^{\Delta R\gamma}$ mice after IMQ. **(b)** H&E-stained **(top)** and Ki-67-stained **(bottom)** sections. Scale bars = 100 µm. Averaged values of epidermal thickness are shown **(right)**. Data are shown as means ± SEMs. ***P < 0.001; 1-way ANOVA with the Bonferroni post hoc test.

IMQ-treated LAT1$^{\Delta R\gamma t}$ mice also displayed decreased infiltration of neutrophils and monocyte-derived inflammatory macrophages compared with IMQ-treated LAT1^{WT} mice **(Figure 5.38a)**. Importantly, lower numbers of dermal CD4^{+} and γδ T-cell populations were observed in the dorsal skin of IMQ-treated LAT1$^{\Delta R\gamma t}$ mice compared with LAT1^{WT} mice **(Figure 5.38b)**.

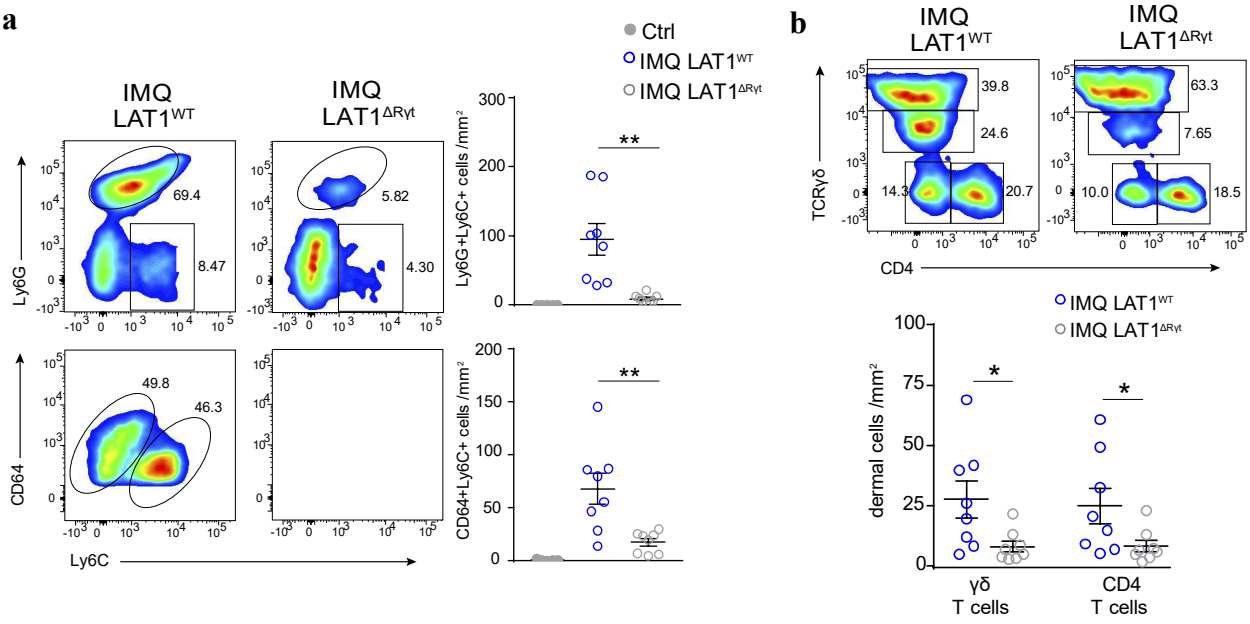

Figure 5.38. IMQ-treated LAT1$^{\Delta R\gamma t}$ mice also displayed decreased cellular infiltration. (a) Skin neutrophils (Ly6G^{+}Ly6C^{+}) **(upper)** and resident (CD64^{+}Ly6C^{-}) versus inflammatory macrophages (CD64^{+}Ly6C^{+}) **(bottom)** were quantified. Absolute numbers of cells are shown **(right)**. **(b)** Dermal CD4^{+} **(bottom and right)** and γδ T cells **(middle and left)** and epidermal γδ T cells **(upper and left)** were analyzed. Absolute numbers of cells are shown **(bottom)**. Data are represented as means ± SEMs. Results of 2 independent experiments are represented as absolute numbers per square millimeter (n = 4) **(a)**. Individual data from one representative experiment of 2 were shown **(b).** *P < 0.05; **P < 0.01; 1-way ANOVA with the Bonferroni post hoc test **(a)** and 2-tailed Mann-Whitney test **(b)**.

Analysis of draining lymphoid cells from IMQ-treated LAT1^{WT} mice showed that IL-17-producing CD27^{-} γδ T cells express LAT1 in contrast to CD27^{+} γδ T cells, which do not secrete IL-17 **(Figure 5.39)**. LAT1$^{\Delta R\gamma t}$ mice showed a reduced frequency of CD27^{-} Tm^{+} γδ T cells and lower Ki-67 expression than IMQ-treated LAT1^{WT} mice **(Figure 5.40)**.

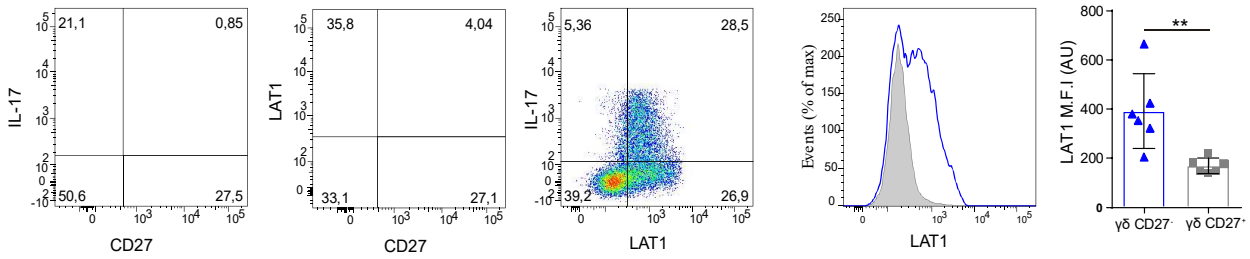

Figure 5.39. IL-17-producing CD27^{-} but not CD27^{+} γδ T cells express LAT1 after IMQ. Dot plots of lymph node γδ T cells T cells from WT mice after IMQ. Expression of LAT1 in CD27^{-}IL-17^{+} cells is shown **(dot plots)** and compared with CD27^{+} γδ T cells **(histograms and bars)**. A representative experiment of 2 individual replicates is shown (n = 5-6). Data are shown as means ± SEMs. **P < 0.01; 2-tailed unpaired Student t test.

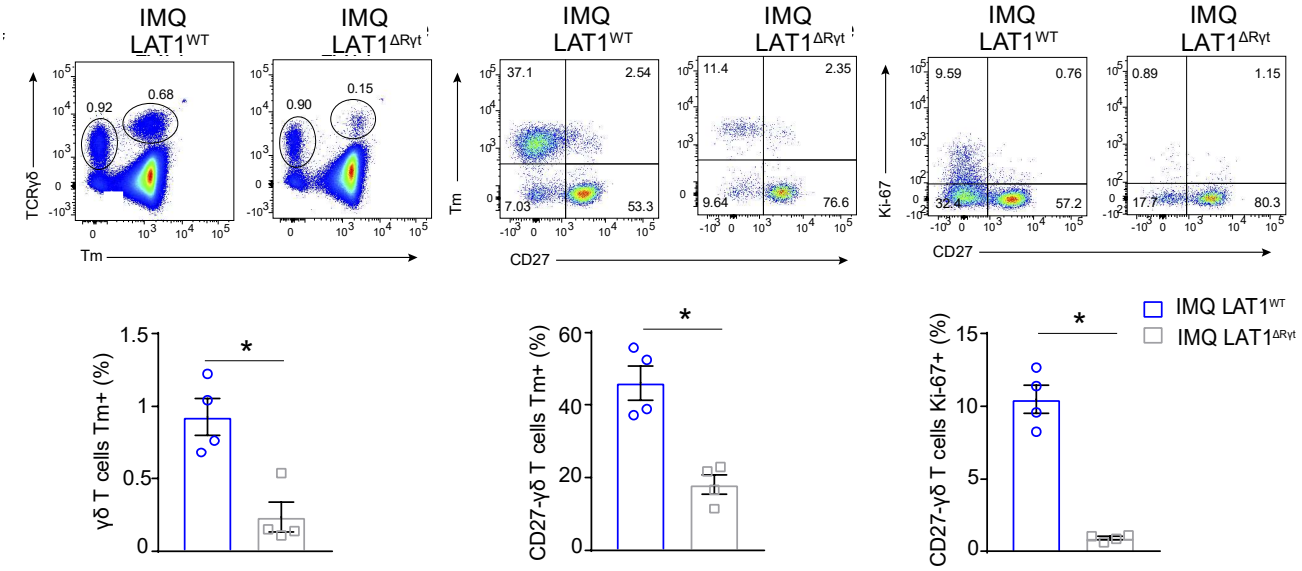

Figure 5.40. IMQ-treated LAT1$^{\Delta R\gamma t}$ mice also displayed lower Ki-67 expression. Dot plots of Tm^{+} γδ T cells from CD3^{+} cells **(left)**, Tm^{+} CD27^{-} cells **(middle)**, and CD27^{-} γδ Ki-67^{+} T cells **(right)** from total γδ T cells are shown. Frequency values are indicated **(bottom)**. Data are represented as means ± SEMs. Individual data from one representative experiment of 2 were shown. *P < 0.05; 2-tailed unpaired Student t test.

Moreover, application of IMQ increased expression of LAT1 in dermal Vγ4^{+} and Vγ4^{-} γδ T cells in LAT1^{WT} mice **(Figure 5.41a)**. Similarly, LAT1 expression was mainly increased in CD27^{-} Vγ4^{+}δ4^{+} T cells detected in dLNs after IMQ application **(Figure 5.41b)**. These results indicate that deletion of LAT1 affected innate and adaptive lymphocyte expansion induced by IMQ, suggesting its potential role as a therapeutic target in skin inflammation.

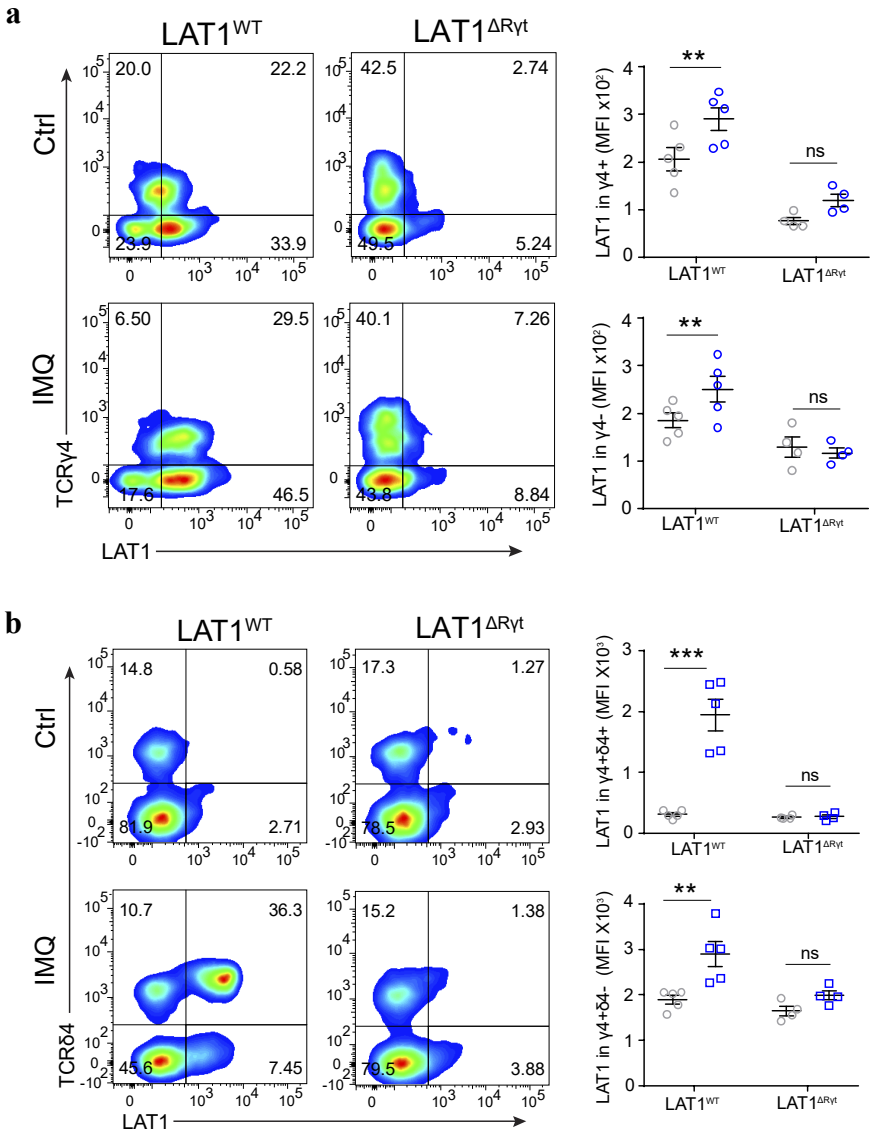

Figure 5.41. IMQ increased expression of LAT1 in dermal γδ T cells. (a) LAT1 expression in Vγ4^{+} **(upper)** and Vγ4^{-} **(bottom)** dermal T cells and **(b)** lymph node Vδ4^{+} **(upper)** and Vδ4^{-} **(bottom)** T cells in normal and IMQ-treated mice. Data are represented as means ± SEMs. Individual data from one representative experiment of 2 were shown (n = 4-5). ns, not significant; **P < 0.01, and ***P < 0.001; 2-way ANOVA with the Bonferroni post hoc test.

In addition, LAT1$^{\Delta R\gamma t}$ mice showed less ear thickness, S100A8/9 expression, and neutrophil and macrophage skin infiltration than LAT1^{WT} mice **(Figure 5.42)** in an alternative model of psoriasis induced by intradermal injection of IL-23.

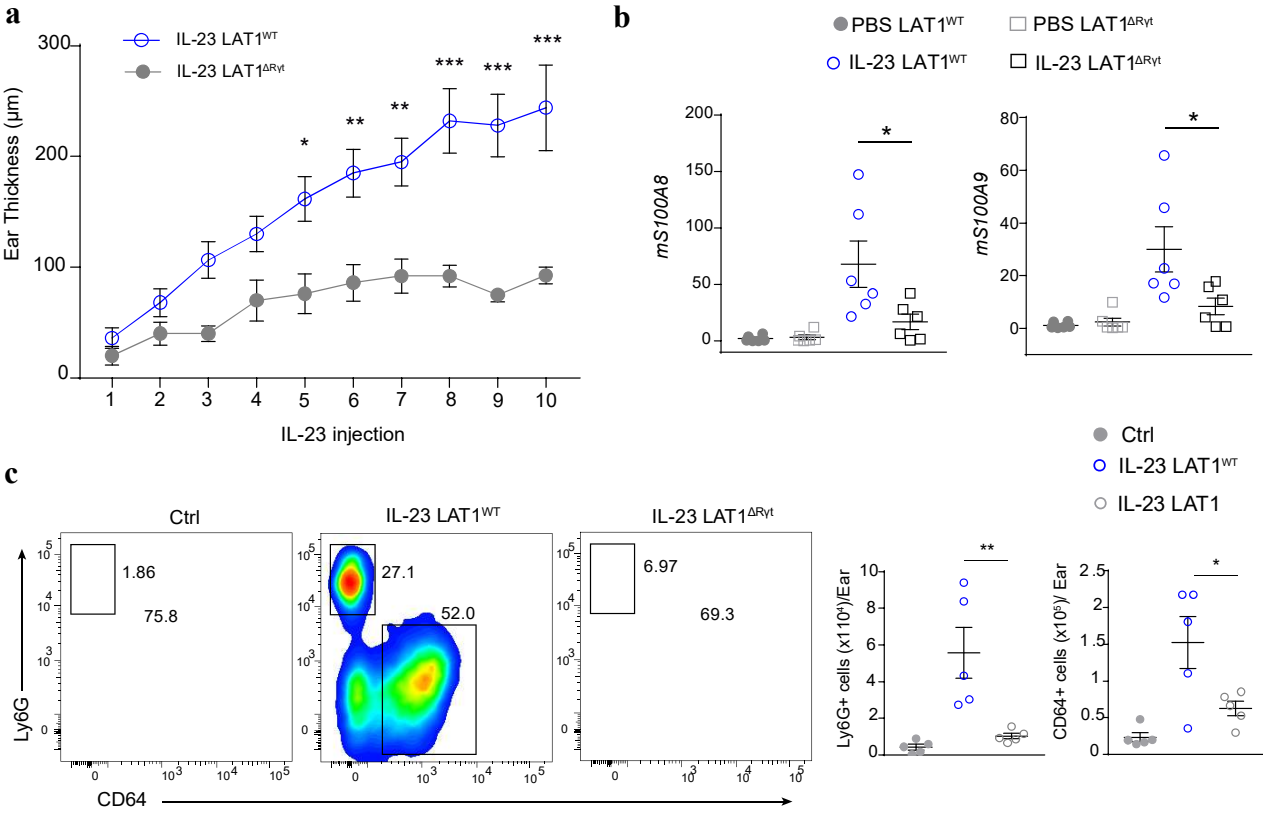

Figure 5.42. LAT1$^{\Delta R\gamma t}$ mice showed less inflammation in IL-23-induced psoriasis model (a) Ear thickness of LAT1 $^{\Delta R\gamma t}$ and LAT1^{WT} mice assessed after IL-23 intradermal injections. **(b)** Transcriptional levels of indicated genes in the skin of mice treated with PBS or IL-23. **(c)** Representative density plots and density values of neutrophils (Ly6G^{+}) and macrophages (CD64^{+}) infiltrating the skin of mice treated or not with IL-23. A representative experiment of 2 individual replicates is shown (n = 5-6). Data are shown as means ± SEMs. ns, not significant; *P < 0.05, **P < 0.01 and ***P < 0.001; 2-tailed unpaired Student t test **(a)** and 1-way ANOVA with the Bonferroni post hoc test **(b, c)**.

5.2.3. Specific deletion of LAT1 in CD4 T cells attenuates psoriasis

Although γδ T cells are essential to develop psoriasis in the IMQ- and IL-23-induced models (24, 175), this population is not the most abundant in patients with psoriasis, in which most of the IL-17-secreting cells are TCRαβ cells (176). To determine whether specific deletion of LAT1 in CD4^{+} T cells controls psoriasis development induced by IMQ, we crossed LAT1$^{fl/fl}$ mice with CD4-Cre$^{+/-}$ mice (177). LAT1 deletion in CD4^{+} T cells was confirmed by using Western blotting and flow cytometry analysis **(Figure 5.43a)**. *In vitro*-activated CD4^{+} T cells from LAT1$^{\Delta CD4}$ mice showed reduced amino acid uptake compared with LAT1^{WT} CD4^{+} T cells **(Figure 5.43b)**. Importantly, the specific inhibitor of LAT1, JPH203, completely blocked amino acid uptake in CD4^{+} T cells, indicating the relevance of this specific essential amino acid transporter in CD4^{+} T cells.

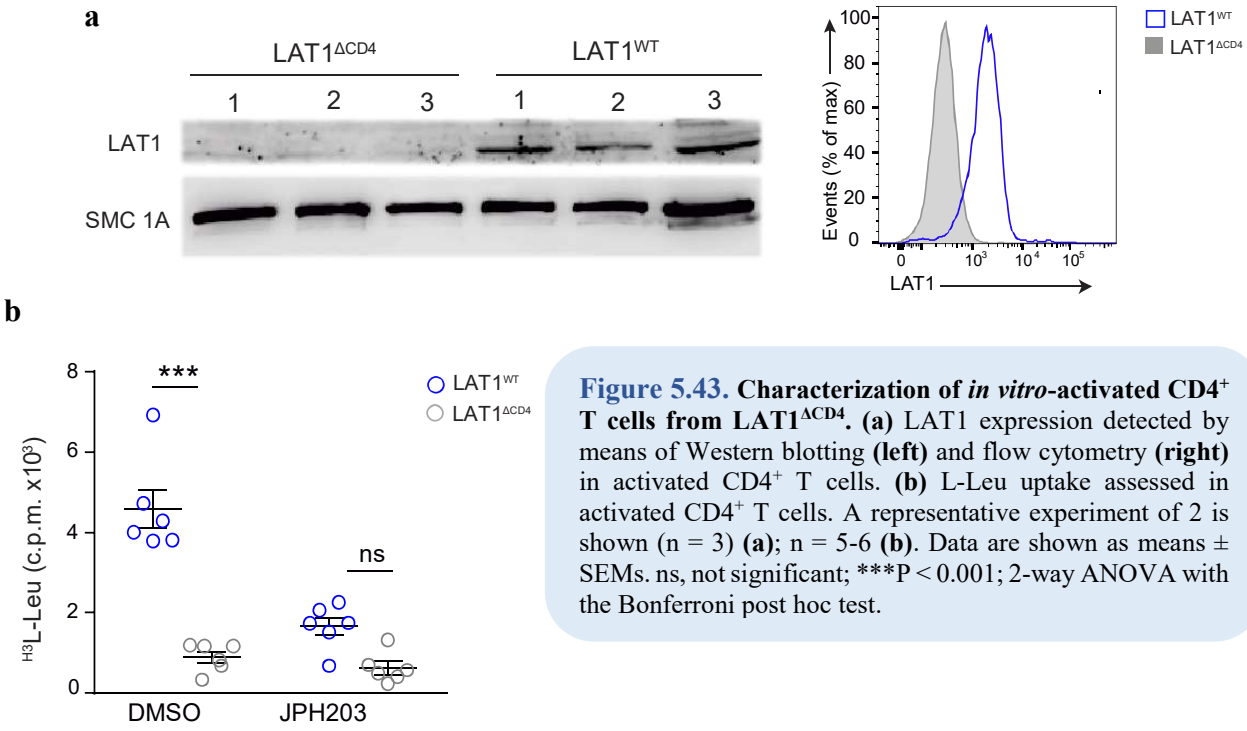

Figure 5.43. Characterization of *in vitro*-activated CD4⁺ T cells from LAT1$^{\Delta CD4}$. **(a)** LAT1 expression detected by means of Western blotting **(left)** and flow cytometry **(right)** in activated CD4⁺ T cells. **(b)** L-Leu uptake assessed in activated CD4⁺ T cells. A representative experiment of 2 is shown (n = 3) **(a)**; n = 5-6 **(b)**. Data are shown as means ± SEMs. ns, not significant; ***P < 0.001; 2-way ANOVA with the Bonferroni post hoc test.

After treatment with IMQ, LAT1$^{\Delta CD4}$ mice displayed a smaller affected area and reduced redness and squamous appearance compared with LAT1^{WT} mice **(Figure 5.44a)**. Histologic assessment revealed reduced epidermal thickness in LAT1$^{\Delta CD4}$ mice **(Figure 5.44b)**.

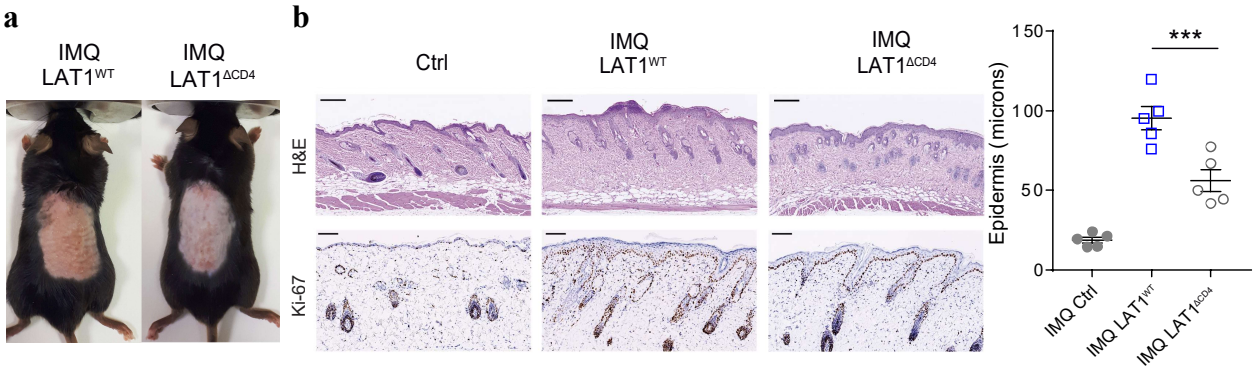

Figure 5.44. LAT1 expression in CD4⁺ T cells contributes to the development of IMQ-induced psoriasis. **(a)** Representative pictures of IMQ-treated LAT1^{WT} and LAT1$^{\Delta CD4}$ mice. **(b)** H&E-stained **(top)** and Ki-67-stained **(bottom)** sections. Scale bars = 100 µm. Averaged values of epidermal thickness are shown **(right)**. Data are shown as means ± SEMs; ***P < 0.001; 1-way ANOVA with the Bonferroni post hoc test.

Numbers of infiltrating neutrophils and CD4⁺ T cells, but not of Ly6C⁺ macrophages and γδ T cells, were attenuated in the skin of LAT1$^{\Delta CD4}$ mice compared to LAT1^{WT} mice **(Figure 5.45a, b)**.

Expression of LAT1 was increased in CD4⁺ T cells after IMQ application **(Figure 5.46a)**. Moreover, CD4⁺ T-cell proliferation was induced by IMQ treatment in the lymph nodes of LAT1^{WT} but not of LAT1$^{\Delta CD4}$ mice **(Figure 5.46b)**.

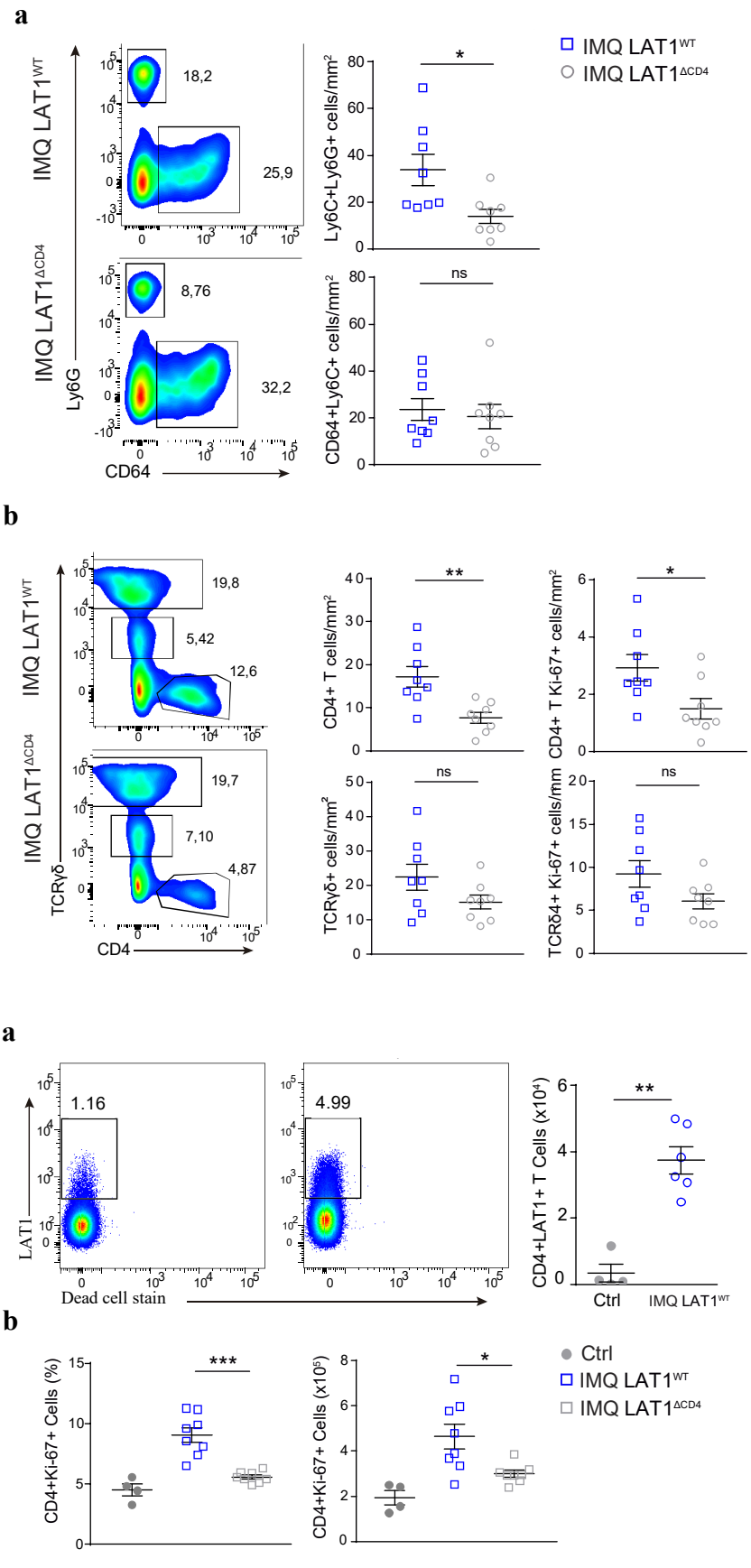

Figure 5.45. LAT1$^{\Delta CD4}$ mice displayed lower numbers of infiltrating neutrophils and CD4$^+$ T cells. (a) Skin neutrophil (Ly6G$^+$; **top**) and macrophage (CD64$^+$; **right**) quantification. **(b)** Dermal CD4$^+$ **(right and bottom)** and γδ **(left and middle)** T cells and epidermal γδ T cells **(left and upper)** were analyzed. Total numbers **(left)** and Ki-67$^+$ dermal CD4$^+$ and γδ T cells **(right)** are shown. A pool of 2 independent experiments is shown. Data are shown as means ± SEMs. ns, not significant; *P < 0.05 and **P < 0.01; 2-tailed unpaired Student t test.

Figure 5.46. IMQ induced the expression of LAT in CD4$^+$ T cells. (a) LAT1 expression in CD4$^+$ T cells in lymph nodes. **(b)** Frequencies and absolute numbers of CD4$^+$Ki-67$^+$ cells in lymph nodes. Data from one of 2 experiments **(a)** or a pool of 2 independent experiments **(b)** are shown. Data are shown as means ± SEMs. *P < 0.05, **P < 0.01, and ***P < 0.001; 2-tailed unpaired Student t test **(a)** and 1-way ANOVA with the Bonferroni post hoc test **(b)**.

As a control, we tested the proliferation of lymph node CD27$^-$ γδ T cells, which remained unaffected when LAT1 was deleted in CD4$^+$ T cells **(Figure 5.47)**.

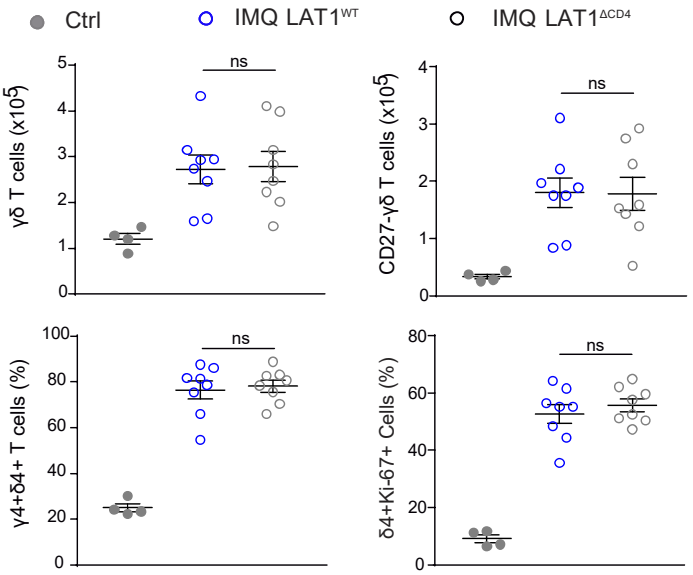

Figure 5.47. The proliferation of CD27⁻ γδ T cells was not affected in LAT1$^{\Delta CD4}$ mice. Absolute numbers of γδ and CD27⁻ γδ T cells and frequency of Vγ4⁺δ4⁺ T cells and Vδ4⁺Ki-67⁺ cells in lymph nodes. A pool of 2 independent experiments is shown (n = 4). Data are shown as means ± SEMs. ns, not significant; 1-way ANOVA with the Bonferroni post hoc test.

Transcriptional expression analysis of purified CD4⁺ **(Figure 5.48)** and γδ **(Figure 5.49)** T cells from the dLNs of mice treated with IMQ indicated that deletion of LAT1 in CD4⁺ T cells specifically controls IL-17 mRNA levels in CD4⁺ T cells but not in γδ T cells. Transcriptional levels of IL-22 and IFN-γ in CD4⁺ and γδ T cells after IMQ application were comparable between both genotypes **(Figures 5.48 and 5.49)**. IL-10 expression was reduced in CD4⁺ T cells from LAT1$^{\Delta CD4}$ mice compared with LAT1^{WT} mice **(Figure 5.48)** but was similar in γδ T cells **(Figure 5.49)**. Expression of LAT1 mRNA levels was also increased by IMQ in CD4⁺ T cells from LAT1^{WT} mice **(Figure 5.48)**.

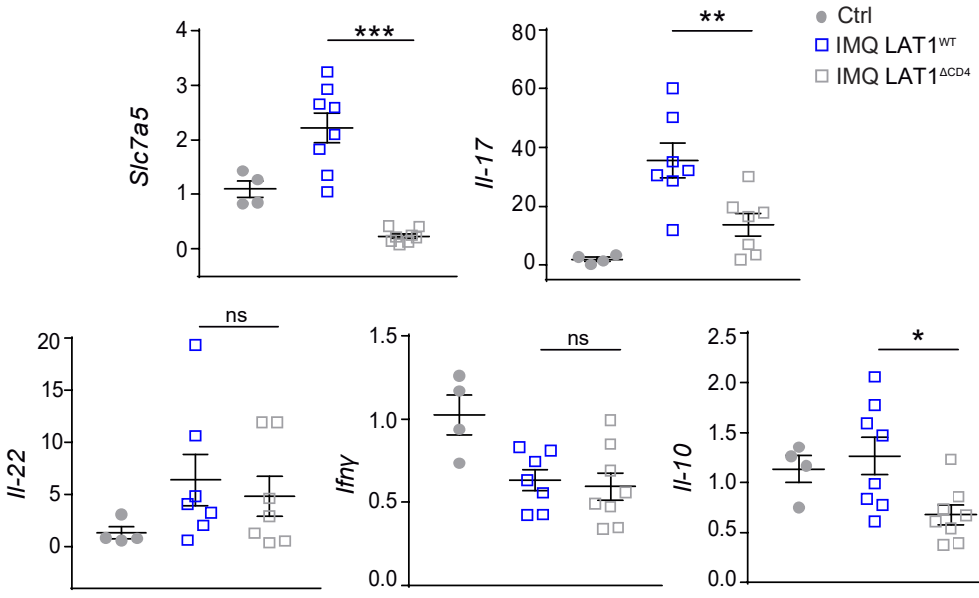

Figure 5.48. The deletion of LAT1 in CD4⁺ T cells specifically controls their IL-17 mRNA levels. Transcriptional expression in CD4⁺ T cells after IMQ. A pool of 2 independent experiments are shown. Data are shown as means ± SEMs. ns, not significant; *P < 0.05, **P < 0.01, and ***P < 0.001; 1-way ANOVA with the Bonferroni post hoc test.

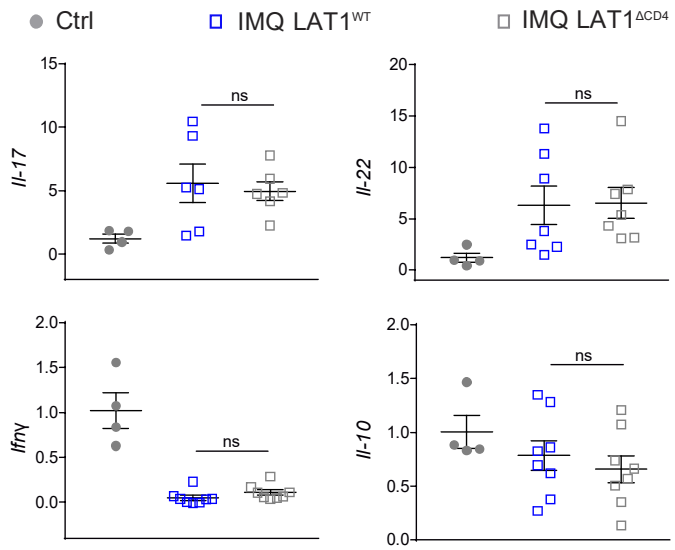

Figure 5.49. Gene expression in γδ T cells was comparable between both genotypes after IMQ application. Transcriptional levels of indicated genes in γδ T cells from lymph nodes. A pool of 2 independent experiments is shown (n = 4). Data are shown as means ± SEMs. ns, not significant; 1-way ANOVA with the Bonferroni post hoc test.

Further stimulation with IL-23 and IL-1β of CD4$^+$ T cells purified from lymph nodes of IMQ-treated mice showed that LAT1$^{\Delta CD4}$ mice displayed lower levels of IL-17 and IL-22 expression **(Figure 5.50a, b)**.

These data indicate that LAT1 controls expansion of CD4$^+$ T cells, as well as their ability to secrete IL-17 and IL-22 in response to IL-23 and IL-1β stimulation.

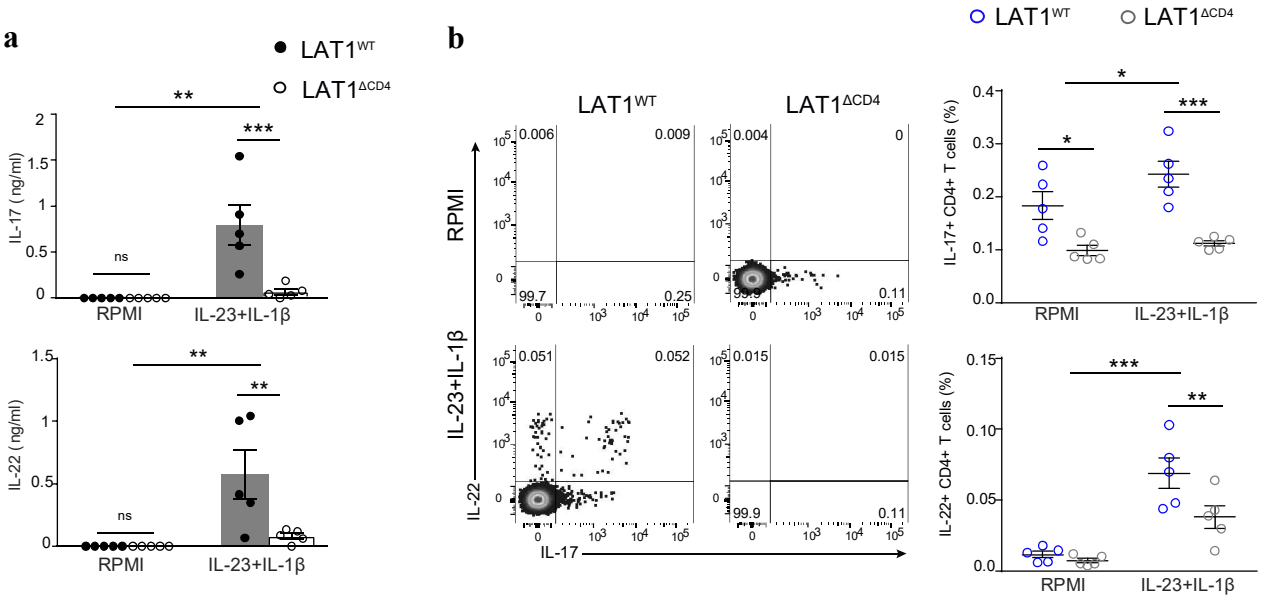

Figure 5.50. LAT1 controls expansion of CD4$^+$ T cells and their capacity to secrete IL-17 and IL-22 in response to IL-23 and IL-1β stimulation. (a) Cytokines level (ELISA). **(b)** Dot plots and frequencies of IL-17$^+$ **(upper)** and IL-22$^+$ **(bottom)** CD4$^+$ T cells obtained from IMQ-treated mice and stimulated with IL-23 plus IL-1β and PMA/ionomycin. Data from one of 2 experiments (n = 4-6) are shown. Data are shown as means ± SEMs. ns, not significant; *P < 0.05, **P < 0.01, and ***P < 0.001; 2-way ANOVA with the Bonferroni post hoc test.

Moreover, naïve CD4$^+$ T cells from LAT1$^{\Delta CD4}$ and LAT1^{WT} mice were differentiated *in vitro* towards the Th17 program by means of addition of IL-6 and TGF-β in combination with IL-23 plus IL-1β. CD4$^+$ T cells from LAT1$^{\Delta CD4}$ mice displayed lower numbers of IL-17$^+$ cells and secreted less IL-17 and IL-22 than cells expressing LAT1 **(Figure 5.51a)**. Human CD4$^+$ T cells from healthy donors were also *in vitro* differentiated towards Th17 with the same cytokine cocktail **(Figure 5.51b)**. After 12 d in culture, stimulation with PMA and ionomycin confirmed that LAT1 inhibition controls secretion of IL-17 and IFN-γ in human CD4$^+$ T cells **(Figure 5.51b)**. These results indicate that LAT1 expression is necessary for human and mouse CD4$^+$ Th1 and Th17 polarization.

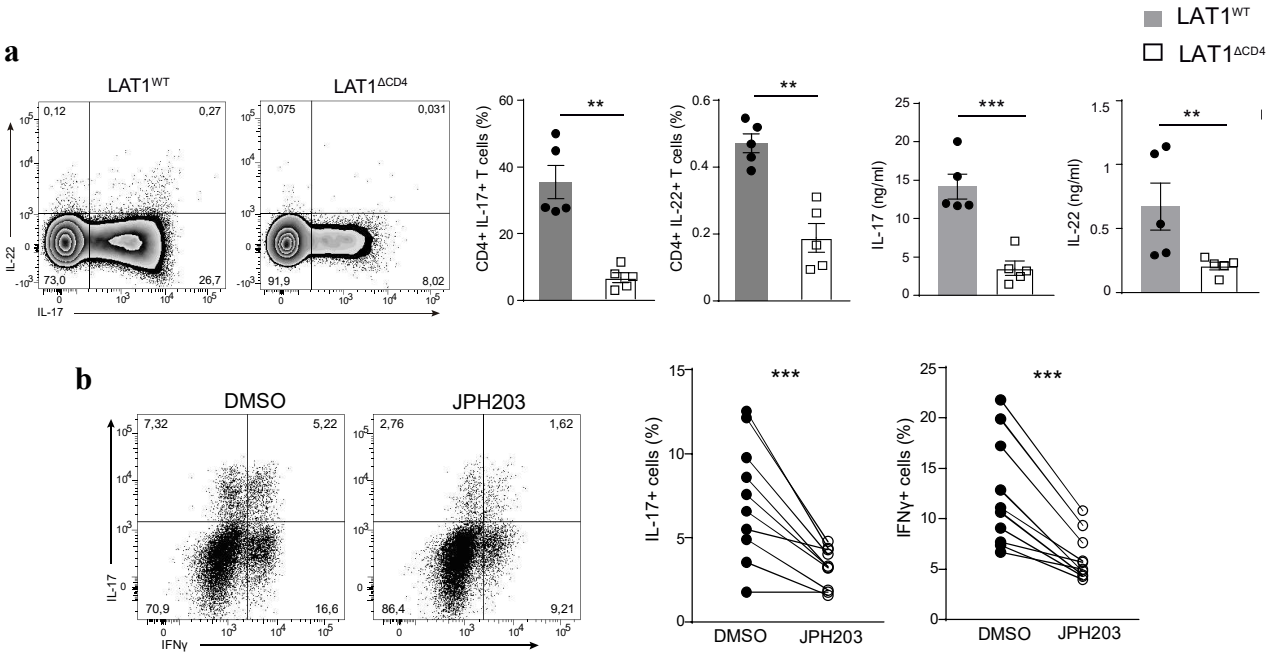

Figure 5.51. LAT1 expression is required for human and mouse CD4$^+$ Th1 and Th17 polarization. (a) Density plots of mouse Th17 cells after PMA/ionomycin stimulation **(left)**. Frequencies of IL-17- and IL-22-secreting cells **(middle)** and cytokine levels (ELISA) **(right)** are shown. **(b)** Human Th17 cells were obtained in the presence of JPH203 or its vehicle. Representative dot plots after PMA/ionomycin stimulation **(left)** and frequencies **(right)** of IL-17- and IFN-γ-secreting cells are shown. A representative experiment of 2 is shown (n = 5-6 **(a)** or n = 10 **(b)**). Data are shown as means ± SEMs. **P < 0.01, and ***P < 0.001; 2-tailed unpaired **(a)** and paired **(b)** Student t tests.

5.2.4. Pharmacologic inhibition of LAT1 prevents IMQ-induced psoriasis in mice

Mice were randomly treated with DMSO (vehicle) or LAT1 inhibitor (JPH203) to ascertain whether pharmacologic inhibition of LAT1 can affect IMQ-induced psoriasis severity. Inhibition of LAT1 during IMQ application caused a marked reduction of the appearance of psoriasis hallmarks, such as redness, neutrophil infiltration, and epidermal thickening **(Figure 5.52)**.

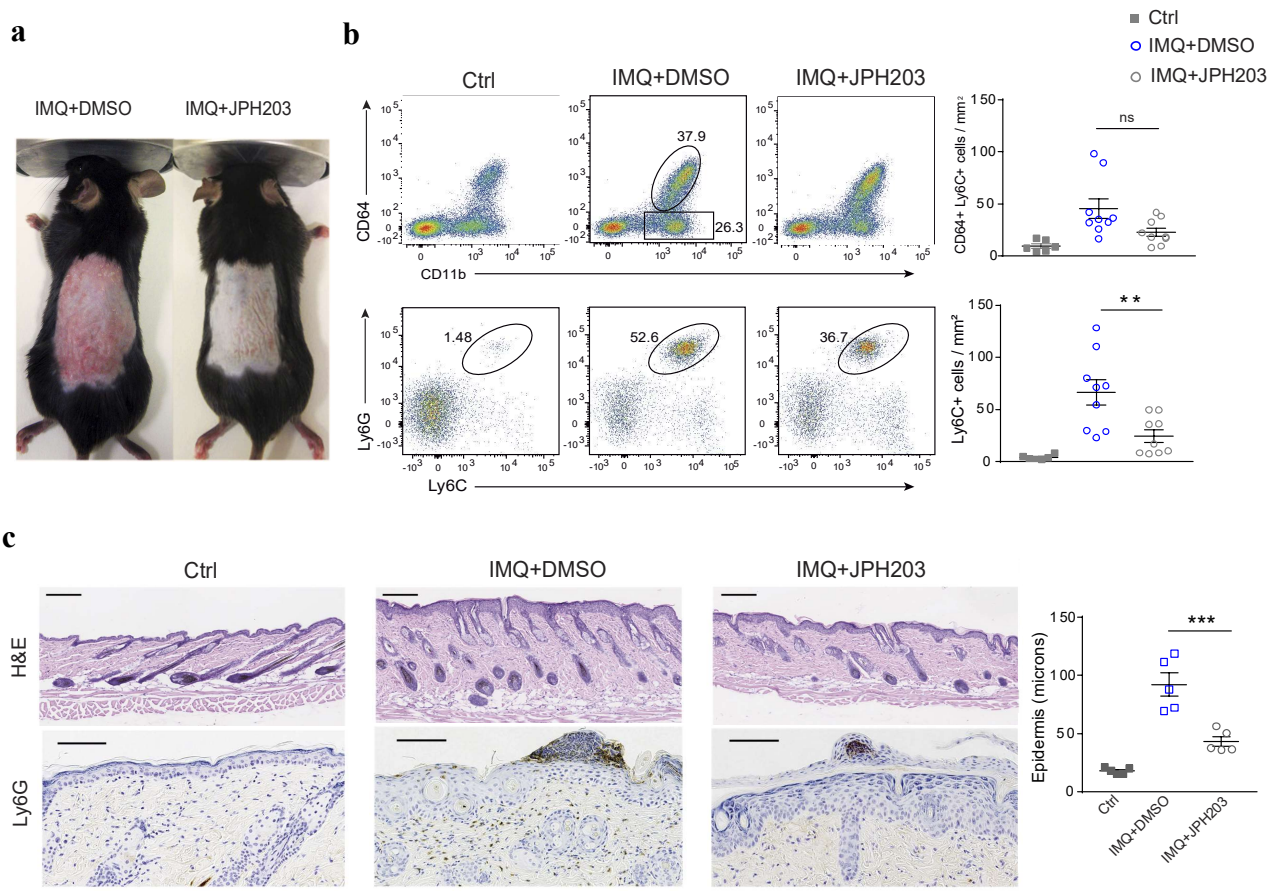

Figure 5.52. The inhibitor of LAT1, JPH203, prevents IMQ-induced skin inflammation. (a) Pictures from mice treated with DMSO or JPH203 and IMQ. **(b)** Dot plots identifying macrophages ($CD64^{+}CD11b^{+}$) **(top)** and neutrophils ($Ly6G^{+}Ly6C^{+}$) **(bottom)** in $CD45^{+}$ and $CD45^{+}CD64^{-}$ populations in the dorsal skin, respectively. Density values of macrophages and neutrophils are shown (**right**). A pool of 2 independent experiments is shown (n = 4-5). **(c)** H&E-stained **(top)** and Ly6G-stained **(bottom)** skin sections. Scale bars = 100 µm. Averaged values of epidermal thickness per mouse are shown in the graphic at **right**. Data are shown as means ± SEMs. ns, not significant; *P < 0.05, **P < 0.01, and ***P < 0.001; 1-way ANOVA with the Bonferroni post hoc test.

To confirm the beneficial effect of JPH203 against psoriasis, we evaluated transcriptional levels of several proinflammatory mediators in the skin **(Figure 5.53)** and dLNs **(Figure 5.54)**. JPH203 treatment prevented the increase in IL-1β, IL-17A, IL-22, S100A8, and S100A9 levels induced by IMQ in the skin **(Figure 5.53)**. Interestingly, reduced transcriptional expression of CCR6 was also detected in the skin of JPH203-treated mice compared with that in DMSO-treated mice, which is consistent with a reduction in skin infiltration by IL-17-releasing cells (178). In contrast, we found no difference in IL-23 and IFN-γ levels **(Figure 5.53)**. These experiments also revealed reduced transcription of IL-17 and IL-22 in skin-dLNs from JPH203-treated mice **(Figure 5.54)**. However, transcriptional levels of IL-10, IFN-γ, CCL5, and CCL20 were comparable in both the JPH203- and DMSO-treated groups. Likewise, increased transcriptional levels of the LAT1 gene (Slc7a5) but not the CD98 gene (Slc3a2) were found in the lymph nodes of IMQ-treated mice supplemented with JPH203 or DMSO compared with the control group **(Figure 5.54)**.

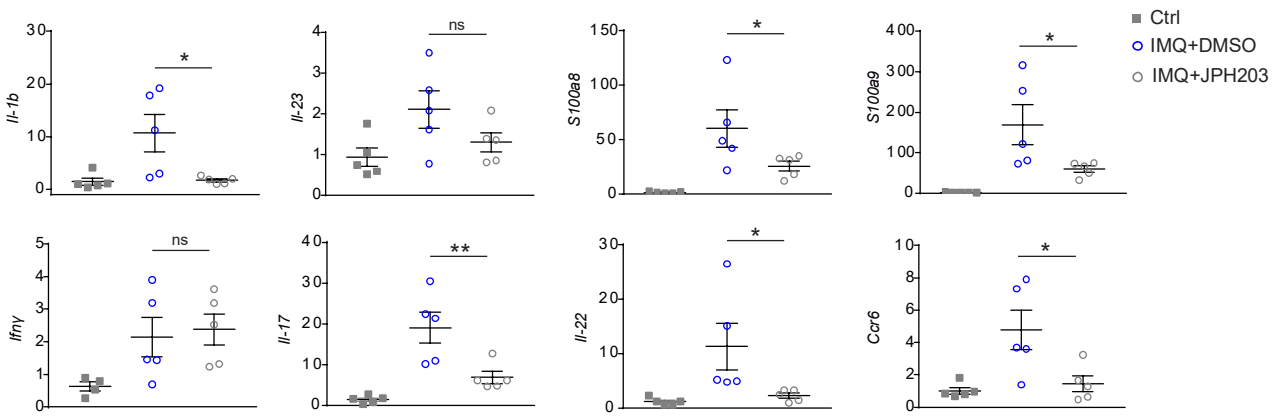

Figure 5.53. JPH203 treatment prevented the increase in several proinflammatory mediators induced by IMQ in the skin. Relative fold induction of indicated genes induced in the skin by IMQ. Data from one of 2 individual experiments are shown (n = 4-5). Data are shown as means ± SEMs. ns, not significant; *P < 0.05 and **P < 0.01; 1-way ANOVA with the Bonferroni post hoc test.

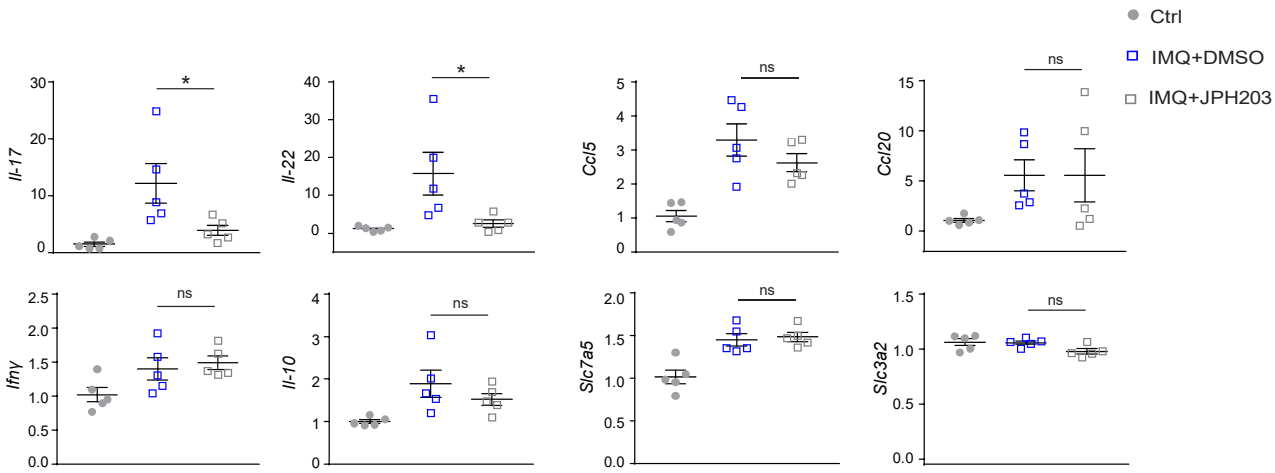

Figure 5.54. JPH203-treated mice showed less transcriptional levels of IL-17 and IL-22 in skin-dLNs. Transcriptional levels of the indicated genes induced in skin-dLN after IMQ. A representative experiment of 2 is shown (n = 4-5 per group). Data are shown as means ± SEMs. ns, not significant; *P < 0.05; 1-way ANOVA with the Bonferroni post hoc test.

Inhibition of LAT1-mediated amino acid transport prevented IMQ-induced expansion of γδ T cells in skin-dLNs, which mostly express γ4 and δ4 chains. A significant reduction in the numbers of $CD27^-$ γδ T cells was detected in mice treated with JPH203 compared with the DMSO-treated group, whereas the population of $CD27^+$ γδ T cells remained unaffected. $CD27^-$ $γ4^+δ4^+$ T cells displayed high levels of Ki-67, indicating that they actively proliferate in the DMSO-treated group, whereas their proliferation was significantly reduced in mice treated with JPH203. These data indicate that LAT1 inhibition controls $CD27^-$ γδ T-cell proliferation **(Figure 5.55)**.

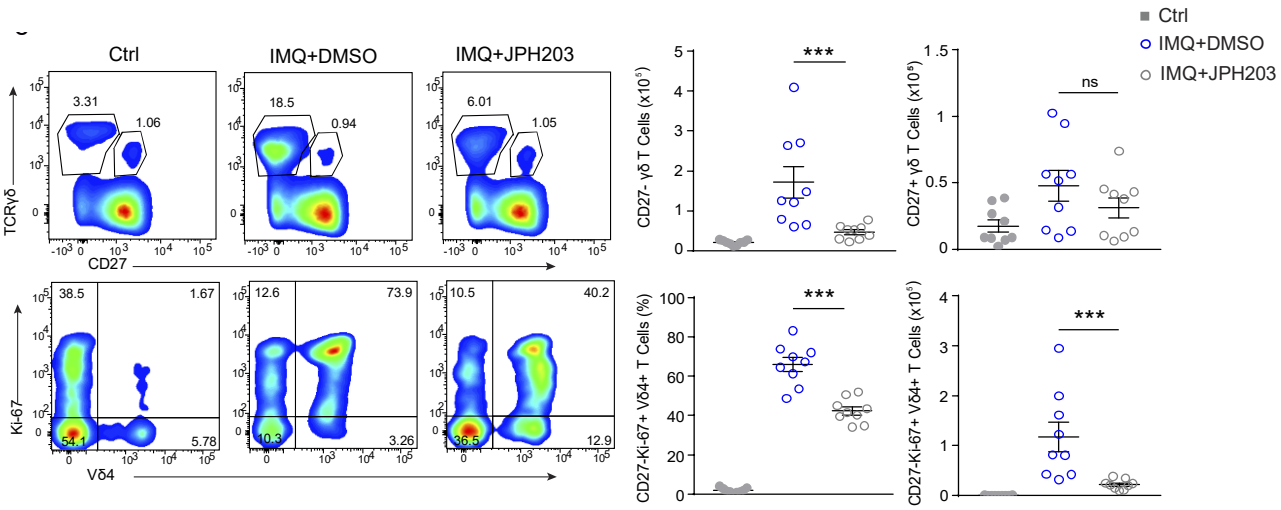

Figure 5.55. LAT1 inhibition controls CD27⁻ γδ T-cell proliferation. Representative density plots for CD27⁻ and CD27⁺ TCR γδ T cells in the CD3⁺ gated population **(upper)** and frequencies of Vδ4⁺Ki-67⁺ cells on gated CD27⁻ γδ T cells **(bottom)** in skin-dLNs. Absolute numbers or frequencies detected in cellular lymph node suspensions are shown per group **(right)**. A pool of 2 independent experiments are shown (n = 4-5). Data are shown as means ± SEMs. ns, not significant; ***P < 0.001; 1-way ANOVA with the Bonferroni post hoc test.

Moreover, the skin of mice treated with JPH203 contained fewer infiltrating Vγ4⁺δ4⁺ T cells than the skin of DMSO-treated mice. Skin-infiltrating Vγ4⁺δ4⁺ T cells were mostly positive for Ki-67 in DMSO-treated mice, indicating their proliferative activity. Also, JPH203 significantly reduced the frequency of Ki-67⁺Vγ4⁺δ4⁺ T cells in the skin **(Figure 5.56)**. In addition to its effect on γδ T cells, JPH203 also controlled the expansion of CD4⁺ T cells (data not shown). In summary, systemic administration of the LAT1 inhibitor JPH203 attenuates the skin response to IMQ by limiting the expression of most proinflammatory mediators as well as the proliferation of CD4⁺ and γδ T cells.

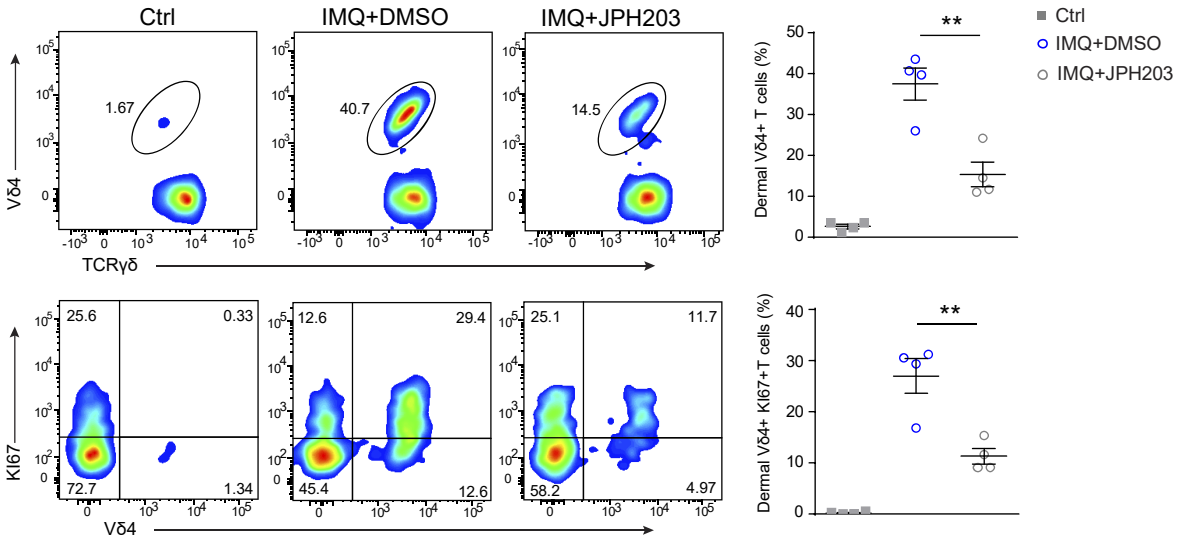

Figure 5.56. JPH203 significantly reduced the frequency of Ki-67⁺Vγ4⁺δ4⁺ T cells in the skin. Density plots of the frequency of Vδ4⁺ and Ki-67⁺Vδ4⁺ T cells from the dermal γδ T-cell population. Frequency values are shown at **right**. A representative experiment of 2 is shown (n = 4-5 per group). Data are shown as means ± SEMs. **P < 0.01; 1-way ANOVA with the Bonferroni post hoc test.

Human γδ T cells can be activated and expanded by phosphorylated antigens, such as zoledronate (163). After 10 d, the presence of Ki-67$^+$ γδ T cells is greater than 85 % (data not shown), but cotreatment with JPH203 significantly prevented their expansion. Western blot analysis of human γδ T cells confirmed expression of the LAT1-CD98 complex **(Figure 5.57)**.

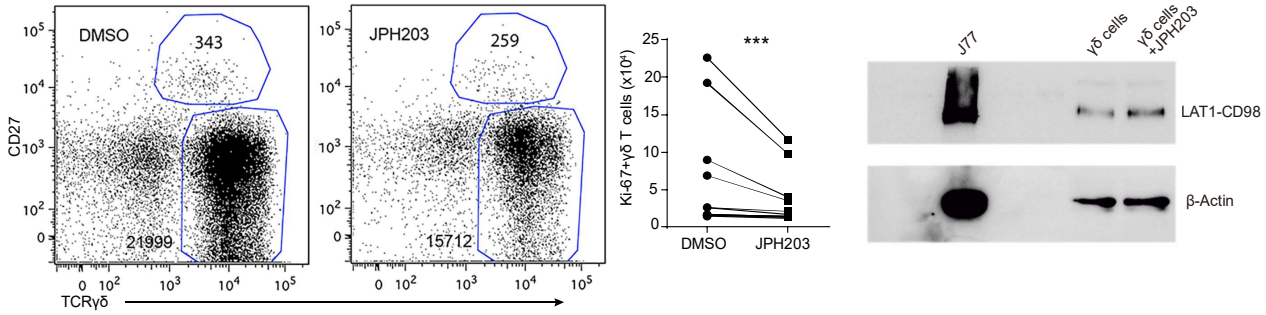

Figure 5.57. JPH203 significantly prevented the expansion of human γδ T cells. Dot plots **(left)** of live human γδ T cells expanded *in vitro* with zoledronate. Values in dot plots indicate total numbers of cells detected in culture after incubation with DMSO or JPH203 normalized by the number of beads. The effect of JPH203 in total γδ T cells obtained from each patient (n = 8 patients) is shown **(middle)**. Representative Western blot of purified γδ T cells from one patient to analyze the LAT1-CD98 amino acid complex is shown **(right)**. ***P < 0.001; 2-tailed paired Student t test.

5.2.5. The LAT1/mTOR axis controls inflammatory responses in the IMQ-psoriasis model

mTOR can sense nutrient availability and it can be activated by several nutrients leading to the phosphorylation of different molecules such as S6 kinase and eukaryotic initiation factor 4E-binding protein 1. This signaling pathway promotes protein synthesis and cell cycle progression and inhibits autophagy. In contrast, under amino acid deprivation, mTOR is dephosphorylated (179). Moreover, PI3K/AKT/mTOR axis is relevant for T cell metabolism and for the induction of aerobic glycolysis and anabolic events in cell growth. Besides, PI3K/mTOR pathway participates in glucose metabolism necessary to the differentiation of effector T cells and to the inhibition of Treg generation (180). To evaluate the role of mTOR activation in the control of CD4$^+$ and Vγ4$^+$δ4$^+$ T-cell expansion in mice with LAT1 deletion or inhibition, we assessed the effect of the mTOR inhibitor, i.e. rapamycin, alone or in combination with JPH203 (JPH203 plus rapamycin) during IMQ challenge. Mice treated with rapamycin or JPH203 plus rapamycin displayed reduced skin inflammation induced by IMQ **(Figure 5.58a)** and reduced epidermal thickness **(Figure 5.58b)**.

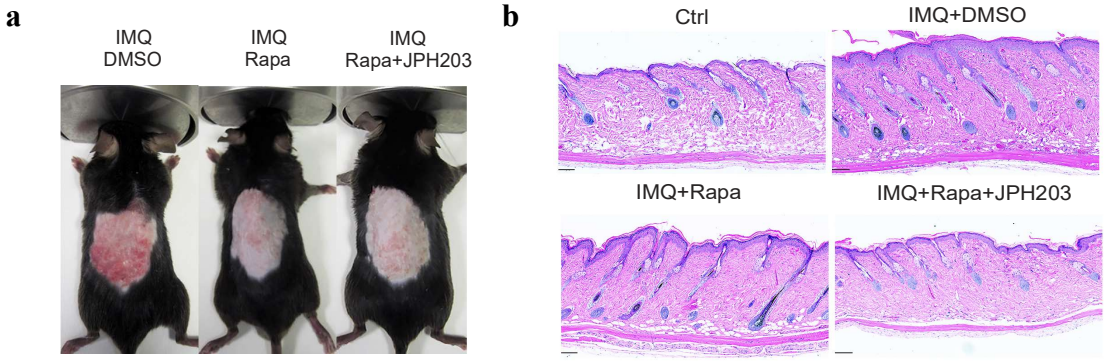

Figure 5.58. LAT1 acts as an upstream regulator of mTOR in the control of IMQ-induced psoriasis. (a) Representative pictures of mice after 4 d of IMQ and treated with DMSO, rapamycin (Rapa), or JPH203 plus Rapa. **(b)** Representative H&E-stained skin sections per group. Scale bars = 100 μm.

Rapamycin and JPH203 plus rapamycin caused a similar reduction in the frequency of Vγ4^{+}δ4^{+} T cells detected in the dLNs **(Figure 5.59)**. The fraction of Vγ4^{+}δ4^{+} T cells expressing Ki-67 was reduced by rapamycin, but no additional inhibitory effect was caused by JPH203 **(Figure 5.59)**. In addition, expansion of CD4^{+} T cells induced by IMQ was prevented by rapamycin or JPH203 plus rapamycin **(Figure 5.60)**. Overall, no additional effect was observed by treatment with JPH203 in mice in which we had already inhibited mTOR signaling in inflammatory cells, indicating that LAT1 might be upstream of the mTOR pathway.

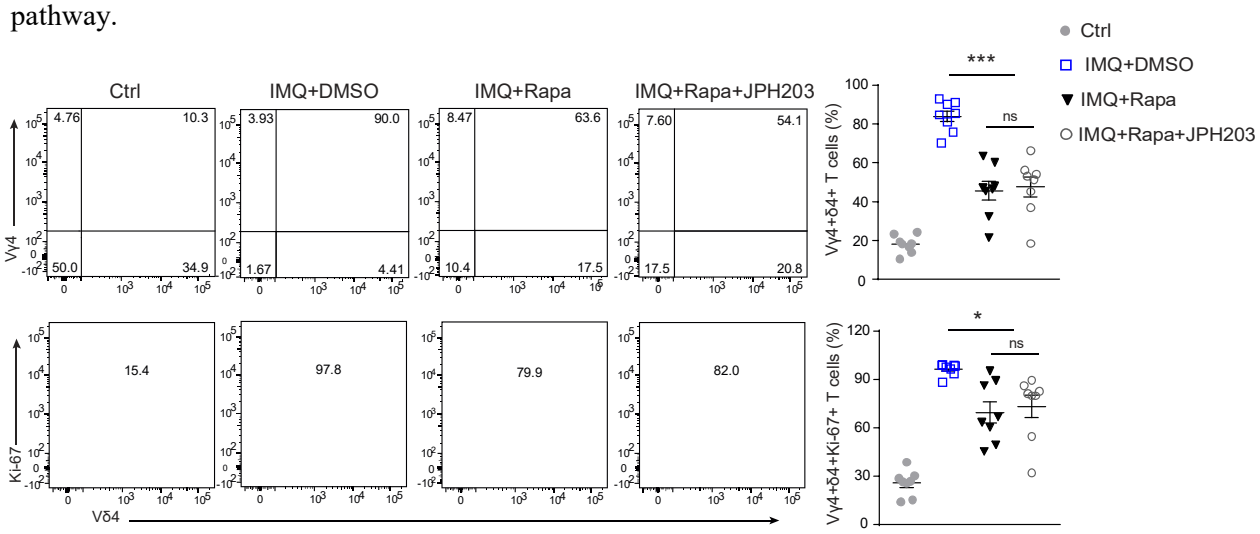

Figure 5.59. JPH203 or JPH203 plus rapamycin (Rapa) showed the same effect in mice. Dot plots of Vγ4^{+}δ4^{+} **(upper)** and density plots of Ki-67^{+}δ4^{+} **(bottom)** from live CD27^{-} γδ T cells quantified in the lymph nodes. Individual values per mouse of frequencies are shown at **right**. A pool of 2 independent experiments are shown (n = 4-6). Data are shown as means ± SEMs. ns, not significant; *P < 0.05, and ***P < 0.001; 1-way ANOVA with the Bonferroni post hoc test.

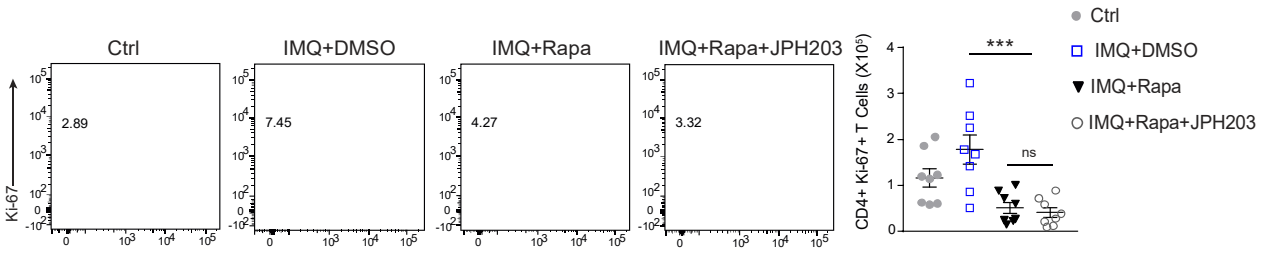

Figure 5.60. JPH203 or JPH203 plus rapamycin (Rapa) prevented the IMQ-induced CD4^{+} T cell expansion. Dot plots of frequencies of Ki-67^{+} cells from CD4^{+} T cells in the lymph nodes. Individual values per mouse of total cell counts are shown at **right**. A pool of 2 independent experiments are shown (n = 4-6). Data are shown as means ± SEMs. ns, not significant; ***P < 0.001; 1-way ANOVA with the Bonferroni post hoc test.

We next assessed the role of the LAT1/mTOR axis in the control of IL-17 and IL-22 secretion after stimulation with IL-23 and IL-1β. Lymph node cells from LAT1$^{\Delta R\gamma t}$ and LAT1^{WT} mice treated with IMQ were used to obtain separate fractions of purified γδ T cells and the remaining lymph node cells. Both fractions, as well as total ear cell suspensions, were *in vitro* stimulated with IL-23 plus IL-1β with or without JPH203 alone or together with rapamycin **(Figure 5.61)**. Cells derived from IMQ-treated LAT1$^{\Delta R\gamma t}$ mice barely responded to IL-23 plus IL-1β stimulation (i.e. they did not secrete IL-17 and IL-22), and the

inhibitory effects observed with JPH203 and JPH203 plus rapamycin were significant only in cells expressing LAT1. In addition, no significant differences were detected between cells treated with JPH203 alone and those treated with JPH203 plus rapamycin **(Figure 5.61)**, further indicating that LAT1 acts upstream of mTOR to control IL-17-related cytokine release.

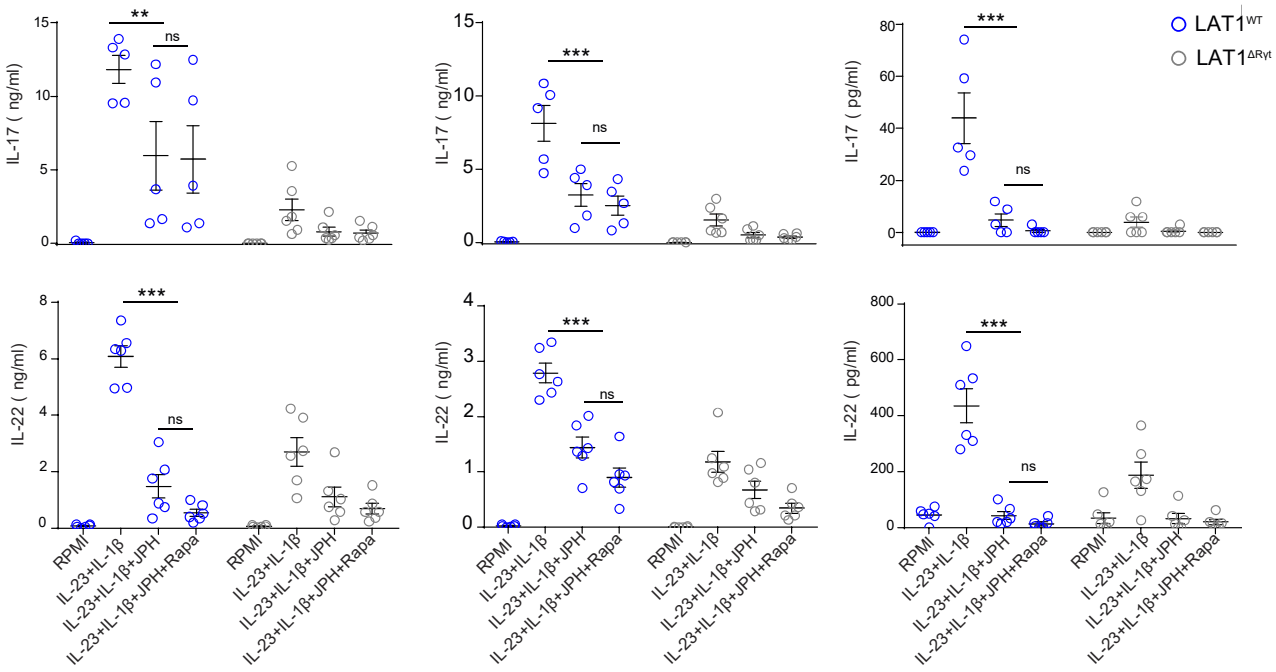

Figure 5.61. LAT1 acts upstream of mTOR to control IL-17-related cytokine release. Purified γδ T cells **(left)**, lymph node cells depleted of γδ T cells **(middle)**, and skin cell suspensions **(right)** of IMQ-treated mice of indicated genotypes were *in vitro* stimulated (24 h) with IL-23 plus IL-1β and incubated with indicated inhibitors. Individual values per mouse of cytokine levels detected in supernatants by means of ELISA are shown. Data from one representative experiment of 2 are shown (n = 4-6). Data are shown as means ± SEMs. ns, not significant; **P < 0.01, and ***P < 0.001; 1-way ANOVA with the Bonferroni post hoc test.

5.2.6. LAT1 regulates the IL-23- and IL-1β-induced PI3K/AKT/mTOR pathway in IL-17-secreting cells

We further explored the mechanism through which LAT1 controls the expansion of immune cells in psoriasis. A previous study proposed that AHR controls γδ T-cell proliferation in response to IMQ (181). To address this, we inhibited LAT1 in $AHR^{+/-}$ and $AHR^{-/-}$ mice. JPH203 treatment dampened $CD27^{-}$ γδ and specifically $V\gamma4^{+}\delta4^{+}$ T-cell expansion in IMQ-treated $AHR^{+/-}$ and $AHR^{-/-}$ mice groups **(Figure 5.62a)**, indicating that the effect of LAT1 inhibition on γδ T-cell proliferation is largely independent of AHR expression. Moreover, JPH203 decreased the frequency and number of IL-17-secreting γδ T cells in both genotypes after IMQ challenge **(Figure 5.62b)**.

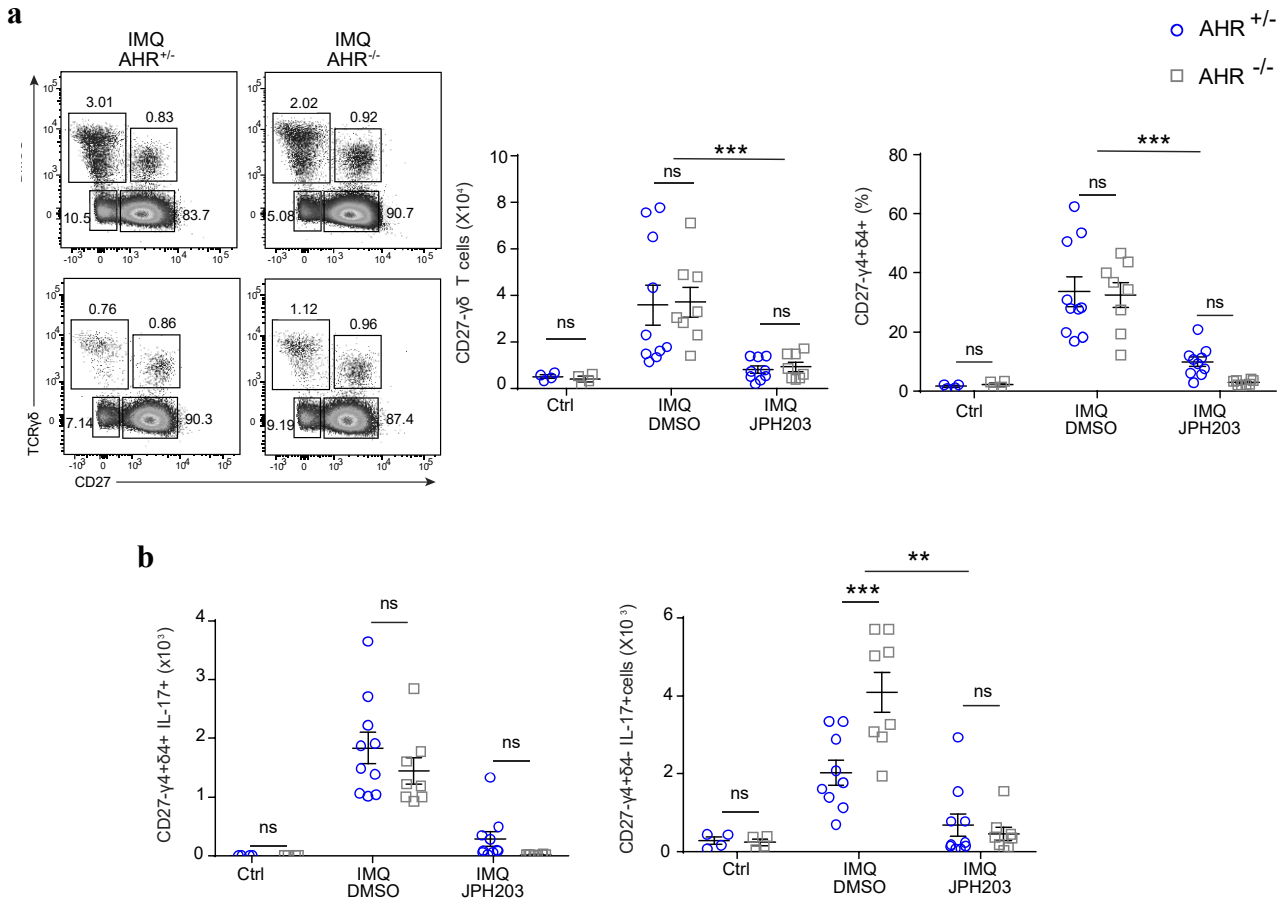

Figure 5.62. LAT1 inhibition controls γδ T-cell proliferation and IL-17 secretion independently of AHR expression. AHR$^{+/-}$ and AHR$^{-/-}$ mice received IMQ in the ear and were injected with Brefeldin A before death. **(a)** Representative dot plots **(left)** and total numbers **(middle)** of CD27^{-} γδ T cells detected in lymph nodes. Frequencies of CD27^{-} Vγ4^{+}δ4^{+} T cells (gated on CD27^{-} γδ T cells) in lymph nodes are also shown **(right)**. **(b)** Total numbers of CD27^{-}Vγ4^{+}δ4^{+} **(left)** and CD27^{-}Vγ4^{+}δ4^{-} **(right)** T cells producing IL-17 are shown. Data pools of 2 independent experiments (n = 4) are represented. Data are shown as means ± SEMs. ns, not significant; **P < 0.01 and ***P < 0.001; 1-way ANOVA with the Bonferroni post hoc test.

To characterize the molecular triggers of LAT1 expression in psoriasis, we assessed the effect of the proinflammatory cytokines IL-23 and IL-1β. Both cytokines play crucial roles in Th17 development (182) and promote extrathymic commitment of IL-17^{+}γδ T cells in the IMQ model (96). LAT1 expression was significantly increased by IL-23 plus IL-1β stimulation in Vγ4^{+} T cells from the lymph nodes of mice treated with IMQ **(Figure 5.63a)**. Stimulation with IL-23 and IL-1β also increased LAT1 expression of CD4^{+}IL-17^{+} T cells differentiated *in vitro* **(Figure 5.63b)**. Moreover, peripheral CD4^{+} T cells from patients with psoriasis expressed high levels of LAT1-CD98 complex when stimulated with IL-23 plus IL-1β compared with cells from healthy donors **(Figure 5.63c)**. These results suggested that LAT1 is essential for the expansion of IL-17-secreting T lymphocytes induced by IL-23 and IL-1β.

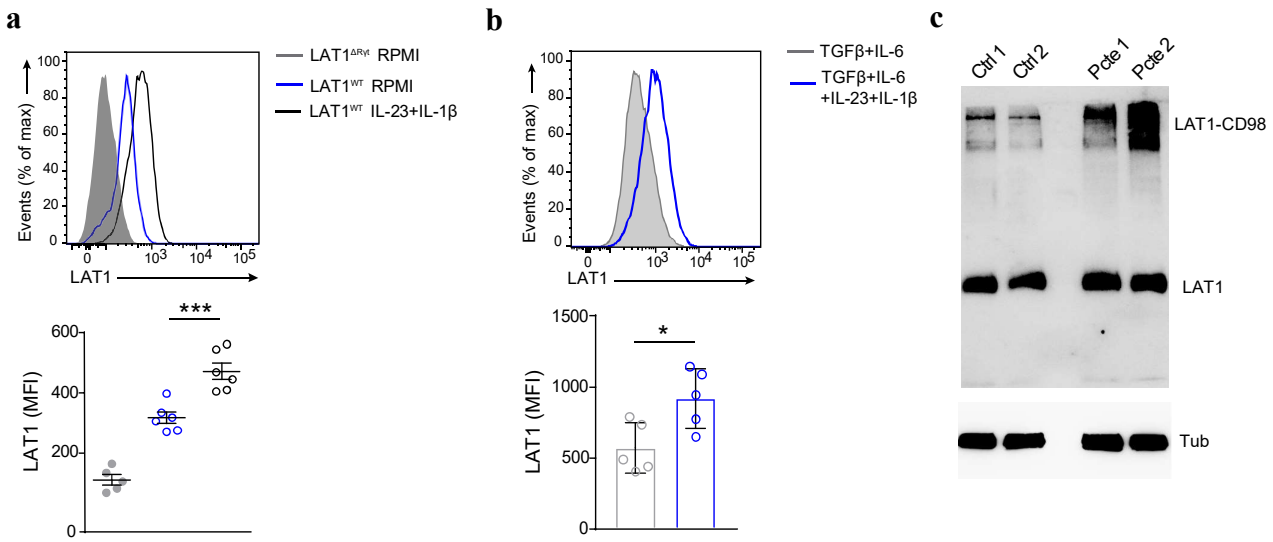

Figure 5.63. IL-23 and IL-1β upregulate LAT1 expression in IL-17-secreting cells. Representative histograms **(upper)** and individual values **(bottom)** of LAT1 fluorescence in Vγ4$^+$ cells from lymph node cells of IMQ-treated mice **(a)** and *in vitro*-skewed Th17 cells from WT mice **(b)** after IL-23 plus IL-1β stimulation. **(c)** Peripheral CD4$^+$ T cells from healthy donors (Ctrl) and patients with psoriasis (Pcte) were *in vitro* stimulated (48 h) with IL-23 plus IL-1β. Expression of LAT1 and the LAT1-CD98 heterodimeric complex determined by using Western blotting are shown. A representative experiment of 2 individual replicates is shown (at least n = 5 **(a, b)** and n = 2 **(c)** samples per group). Data are represented as means ± SEMs. *P < 0.05 and ***P < 0.001; 1-way ANOVA with the Bonferroni post hoc test **(a)** and the 2-tailed paired Student t test **(b)**.

IL-23 and IL-1β also induce activation of the mTOR pathway, as assessed by an increase in P-S6 detection, in γδ T cells. Remarkably, the combination of IL-23 plus IL-1β potentiates the effect of each cytokine alone on P-S6 activation in both γ4$^+$ and γ4$^-$ T cells **(Figure 5.64)**.

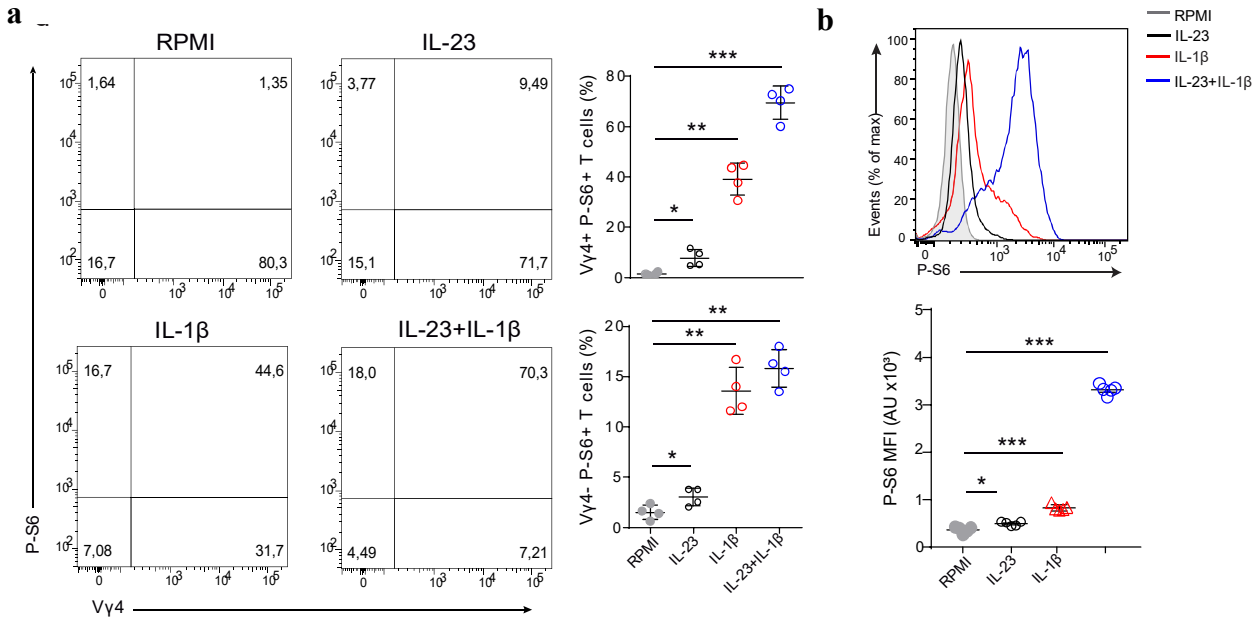

Figure 5.64. IL-23 and IL-1β induce mTOR activation in IL-17-secreting cells. (a) Total lymph node γδ T cells from IMQ-treated WT mice were stimulated (24 h) with IL-23, IL-1β, or both. Dot plots **(left)** and frequencies **(right)** of P-S6 expression in γ4$^+$ **(upper)** and γ4$^-$ **(bottom)** cells are shown. **(b)** Histograms **(upper)** and values of fluorescence intensity for P-S6 expression **(bottom)** in the total fraction of CD27$^-$γ4$^+$ T cells stimulated as in **(a)**. A representative experiment of 2 individual replicates is shown (at least n = 5 samples per group). Data are represented as means ± SEMs. *P < 0.05, **P < 0.01 and ***P < 0.001; 1-way ANOVA with the Bonferroni post hoc test.

Stimulation with IL-23 plus IL-1β induced expansion of Vγ4^{+} cells but only when LAT1 was expressed. However, expansion of Vγ4^{+} T cells from LAT1^{WT} mice is reduced by addition of either mTOR or LAT1 inhibitors. These inhibitors had no effect on cells from LAT1$^{\Delta R\gamma t}$ mice **(Figure 5.65)**. Finally, the combination of mTOR and LAT1 inhibitors had no additive effect, further confirming that LAT1 and mTOR are likely part of the same signaling pathway in these cells.

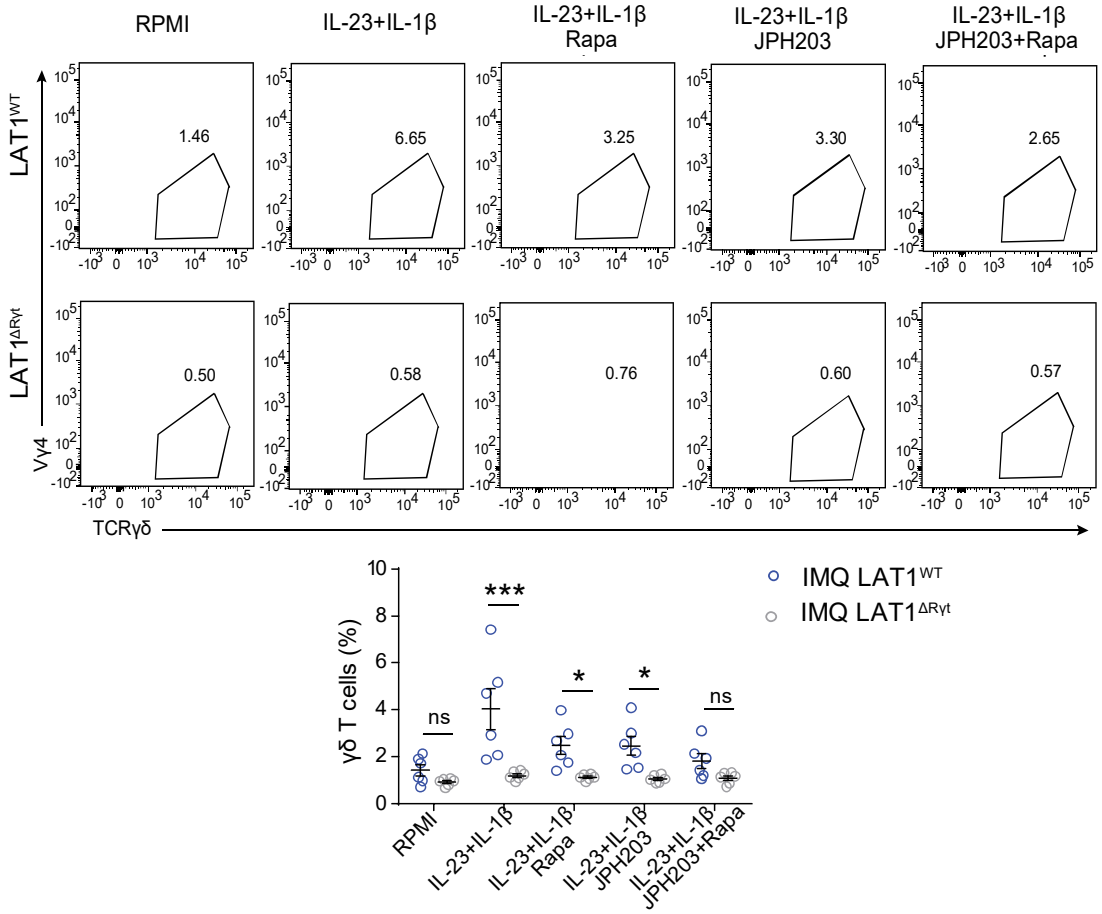

Figure 5.65. The expansion of Vγ4^{+} T cells from LAT1^{WT} mice is reduced by addition of rapamycin (Rapa) or JPH203. Lymph node cells from IMQ-treated mice were stimulated *in vitro* (48 h) with the indicated cytokines and inhibitors. Dot plots **(upper)** and individual values **(bottom)** of γδ T-cell frequencies from CD3^{+} cells are shown. A representative experiment of 2 individual replicates is shown (at least n = 5). Data are shown as means ± SEMs. ns, not significant; *P < 0.05 and ***P < 0.001; 2-way ANOVA with the Bonferroni post hoc test.

The effects of IL-23 plus IL-1β stimulation in BrdU incorporation and S6 phosphorylation in Vγ4^{+} T cells were completely blocked by PI3K inhibitor (Ly294002) or by amino acid depletion **(Figure 5.66)**. Cells from LAT1$^{\Delta R\gamma t}$ mice did not display mTOR activation or proliferation under any of the conditions assayed **(Figure 5.66)**. These data indicate that IL-23 and IL-1β trigger LAT1 expression in Vγ4^{+} T cells and Th17 cells, which promotes PI3K/AKT-induced mTOR signaling to control their expansion and IL-17 secretion.

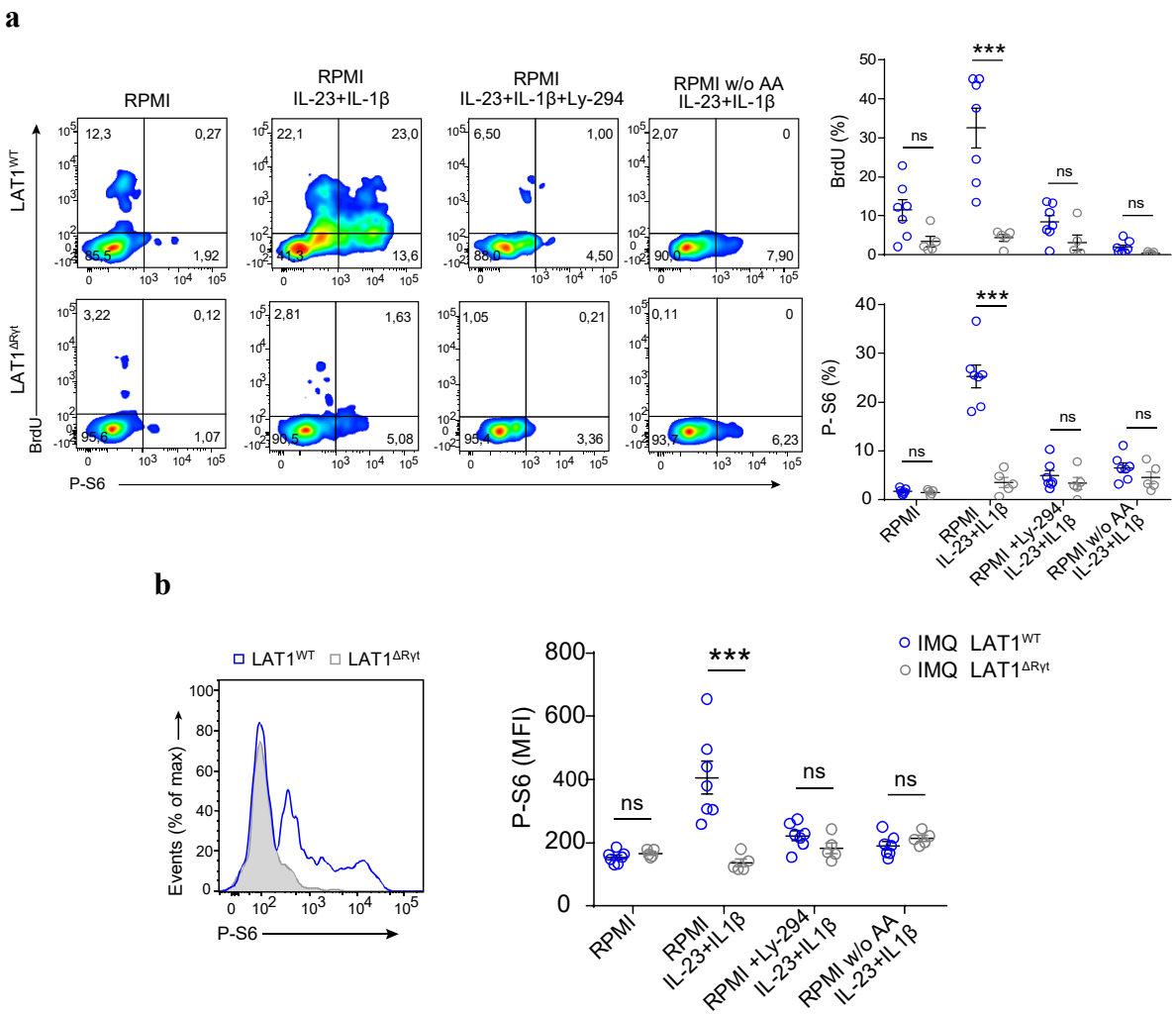

Figure 5.66. LAT1 is required to activate the PI3K/AKT/mTOR pathway induced by IL-23 and IL-1β to promote proliferation of IL-17-releasing cells. (a) Density plots **(left)** and frequencies **(right)** of $BrdU^+$ and $P\text{-}S6^+V\gamma4^+$ cells obtained and stimulated. **(b)** Histogram of P-S6 expression **(left)** and individual values of P-S6 fluorescence **(right)** in $V\gamma4^+$ cells are shown. A representative experiment of 2 individual replicates is shown (at least n = 5). Data are shown as means ± SEMs. ns, not significant; *P < 0.05 and ***P < 0.001; 2-way ANOVA with the Bonferroni post hoc test.

DISCUSSION

6. DISCUSSION

Inflammatory skin diseases are highly prevalent and complex defined by modifications in the epithelium and in T cell immunity (183). ACD depends on the rapid activation of specific T cells and the cytokine and chemokine secretion after the penetration of a hapten through the epidermis and its recognition by DCs (63, 64). Furthermore, the pathogenesis of psoriasis involves the interplay between the immune system and KCs, the presence of susceptibility loci associated with psoriasis, autoantigens and environmental factors (87). Genetic studies aimed at identifying new genetic pathways associated with psoriasis risk demonstrate that alterations in metabolic pathways positively correlate with increased psoriasis risk (184).

Due to the complexity of these inflammatory skin diseases, patients with ACD or psoriasis are treated with different therapies described along the introduction (65, 85, 86, 185), but further studies are necessary to improve the diagnosis and to generate alternative drugs. Therefore, in this thesis work we have addressed the role of two immunoregulatory molecules, Gal-1 and LAT1, in ACD and psoriasis mouse models, respectively, to expand the current knowledge about the mechanisms involved in the development of these skin disorders. We demonstrate that the endogenous expression of Gal-1 in effector and memory $CD8^+$ T cells plays an essential role in the development of CHS model. In the absence of this galectin, we found that mice develop an exacerbated CHS inflammation response mediated by an increase of $CD8^+$ and IL-17-secreting γδ T cells. In regard to psoriasis, we observed the increased expression of LAT1 in psoriatic lesions in KCs and infiltrating lymphocytes. Pharmacologic inhibition of LAT1 efficiently blocks skin inflammation induced in mice by IMQ application. The anti-inflammatory effects of LAT1 inhibitor can be recapitulated by genetic deletion of LAT1 in lymphocytes, including RORγt-expressing cells, such as γδ T cells and Th17 cells.

6.1. MOUSE MODELS OF ACD AND PSORIASIS

There are different methods, depending on the type of hapten, to induce CHS model. Haptens are a group of small molecules including a reduced number of strong agents involved in mouse models and a huge quantity of weak haptens that can induce human ACD (66, 186). In addition to ACD, these molecules have been typically used to investigate the inflammatory bowel disease in mouse models (187). Moreover, the application of a hapten promotes viral wart regression (188, 189), autoimmune responses and antitumor immunity (186, 190, 191). The diverse effects of cytokines associated to CHS could be due to the differences in the used hapten, model and also the microbiota. The cytokines produced in the elicitation phase depend on the nature of the hapten, for instance, TNCB, OXZ and DNFB promote Th1 response while FITC induces Th2 response (55, 186). Moreover, some authors demonstrate that TLR4 is stimulated when the exposure to the hapten takes places few times leading to a Th1 response. In contrast, Th2 response occurs when the contact with the hapten is prolonged and repeated, and stimulates TLR2. This hypothesis is named as “Hapten Atopy Hypothesis” (192). In agreement with this postulate, in chronic hapten exposure, a decrease of Th1 cytokines (TNFα, INFγ, IL-2, and IL-12) but an increase of Th2 cytokines (IL-4, IL-5, and IL-13) and Treg associated cytokines (IL-10) occurs (186, 193). On the other hand, some authors postulate that microbiota haptenation (bacterial proteins or lipids) could produce different CHS

responses due to the diverse response of the immune compartment, but this view needs experimental demonstration (186). CHS induction exerts alterations in the gut microbiota structure and its composition determines the severity of CHS features. Some foods components and prebiotics attenuate CHS response in mice (194, 195).

There are different mouse models of psoriasis, which include transgenic mice, spontaneous or induced mouse models, T cell-based models and xenografts. In this thesis work, we have applied two commonly used psoriasis-inducing models, IMQ topical treatment and IL-23-injection (110, 112). Although both models have been criticized due to the effect associated with the vehicle of the cream or the IL-23, respectively, they are typically used because they induce skin inflammation in mice with features similar to human psoriatic plaques. These features include epidermal hyperplasia and accumulation of inflammatory cells (110, 112). In mice skin, dermal γδ T cells are the main population that expresses IL-23R in homeostasis (24). Besides, both IMQ and IL-23 psoriasis mouse models present another disadvantage, their dependence on γδ T cells (24, 175), because the relevance of this T cell population is not certainly determined in humans and their frequency is lower in human skin than in mouse skin (176, 196, 197).

6.2. GAL-1 AND NON-LYMPHOID CELLS

Although it is well known that CHS model is mainly mediated by T cells, other immune cell players are also involved in the development of this disorder (185). KCs are involved not only in the physical blockade of external agent penetration but also in the recognition of the haptens. Some patients with alteration in filaggrin gene present ACD to nickel because this protein allows the properly establishment of the cornified cell layer (185, 198). Additionally, KCs promote the increase of Treg cells by expressing the receptor activator for NF-κB ligand (RANKL) that can interact with RANK expressed in LCs. UV-radiation and vitamin D3 are two of the major inducers of RANKL (199). Vitamin D3 therapy is used in psoriatic patients (200, 201). ACD patients and CHS model showed reduced clinical manifestations when are treated with UV-radiation and vitamin D3 which increase RANKL expression in KCs (199, 202, 203). Furthermore, KCs secrete IL-10, which is a immunosuppressive cytokine, and therefore may control the progression of CHS (204). *In vivo* experiments with rats demonstrated that human Gal-1 exerts a positive effect on skin wound closure 21 d after surgery (205). Studies with HaCaT cells showed that Gal-1 induces the migration of KCs (206). However, previous studies in our laboratory demonstrated that human KCs do not express Gal-1 (207). In addition, our studies showed that murine KCs do not express Gal-1 either in homeostasis or after hapten reaction. Although the relevance of KCs in ACD is well described, the role of Gal-1 in KCs in the context of CHS model can be ruled out.

In the skin there are many types of DCs, but in the epidermis layer LCs are the solely type of DCs. Two types, langerin$^+$ and langerin$^-$ dermal DCs are found in the dermis (185). In CHS model, DCs play an essential role because they load the antigen in the periphery and then migrate to the lymph nodes to present it to naïve and memory T lymphocytes (185, 208). At low concentration of the hapten, LCs are the unique APCs but, at higher concentration of the hapten, it can both diffuse through the epidermis or be presented

by dermal DCs (langerin$^+$ and langerin$^-$) in the dermis to develop CHS model. Thus, additionally to LCs, both dermal DCs exert a critical function in the sensitization phase of CHS, in particular at high concentrations of the hapten (208-210). Therefore, LCs are one of the first APCs that can interact with haptens but their role in CHS is not clear because of the apparent contradictory results found in the literature. *In vivo* experimentation with Langerin-DTR mice demonstrates that the absence of LC dampens CHS reaction but there is a functional redundancy between LCs and langerin$^+$ DCs (209, 211, 212). Other *in vivo* experiments using huLangerin-Cre MHC-II^{flox} and huLangerin-Cre IL-10^{flox} showed that mice lacking LCs develop more CHS response because LCs can inhibit the hapten-induced responses due to the interaction between these cells with CD4$^+$ T cells and the IL-10 secretion. Thus, the LC activation state determines their immunological function depending on the hapten dose and nature, and the used experimental model (185, 208, 210, 211, 213).

Additionally, Gal-1 not only induces the secretion of different cytokines such as IL-6 and TNFα by monocyte-derived DCs (MDDCs), but also it can activate human MDDCs to become mature DCs with an up-regulated migration. Thus, Gal-1 may play an important role in the initiation of immune response because can promote the DC maturation and migration through the extracellular matrix *in vitro* (214-216). On the other hand, Gal-1 promotes the generation of tolerogenic DCs involving IL-27 and IL-10 mechanisms (217). Moreover, extracellular matrix deposited Gal-1 inhibits the migration of immunogenic DC across the extracellular matrix and lymphatic endothelial cells but does not exert any effect on the tolerogenic DCs tissue emigration, being an anti-inflammatory mechanism of Gal-1 (218). Some authors postulate that in the initial inflammation the huge Gal-1 concentration could increase immune activation, but when the inflammation decreases, as well as the Gal-1 concentration, its anti-inflammatory effect could appear (215). In psoriasis patients, LCs and dermal DCs present a decreased expression of Gal-1 (219). Concerning ACD, the overexpression of Gal-1 in transfected DCs promotes the induction of the sensitization phase in CHS, but conversely, induces the inhibition of the elicitation phase caused by the apoptosis of activated T cells. DCs and their overexpression of Gal-1 could be a mechanism to differentially control the two phases of CHS (220). Our data show a delay in the migration of DCs from the skin to the lymph nodes at 48 h, in the absence of Gal-1. However, this effect of Gal-1 vanished at 72 h and Gal-1$^{-/-}$ mice showed an elevated inflammatory response due to an increased CD8$^+$ T cell response. Thus, we consider that the delay of dendritic cell migration in Gal-1$^{-/-}$ mice does not appear to be relevant in our results of OXZ-induced model.

Other non-lymphoid cells involved in CHS are neutrophils, which are required to both phases of the CHS model (221). The anti-inflammatory effect of exogenous Gal-1 on cellular recruitment and adhesion has been characterized in polymorphonuclear cells in *in vitro* and *in vivo* experimental models (222, 223). Gal-1 has a dual role depending on the presence or not of an inflammatory event. *In vitro* experiments with rGal-1 demonstrated that Gal-1 can promote the migration of human neutrophils (136). In an ocular inflammation mouse model, administration of rGal-1 has an anti-inflammatory role diminishing the migration of leukocytes into ocular tissues due to the imbalance in adhesion molecule expression (123). Moreover, in a paw edema model, the administration of Gal-1 to WT mice decreases neutrophil

recruitment, inhibiting the first phase of edema, similar to the effects of endogenous Gal-1 (137). In relation to skin inflammatory diseases, the treatment with rGal-1 does not promote a reduction of neutrophils in blood, opposite to that observed in the skin in AD model (63). However, the function of Gal-1 in neutrophils in the OXZ-induced CHS model is unknown. Our results demonstrate that the neutrophilic infiltration is similar after the sensitization phase but increases after CHS model in absence of Gal-1. Thus, it is possible that, as in other inflammatory situations, Gal-1 could dampen neutrophil migration.

In addition, mast cells are relevant in the induction of CHS because can rapidly release pro-inflammatory mediators and promote the migration of DCs to lymph nodes (69). However, mast cells are able to limit the inflammatory response in CHS mouse model 15 d after the challenge by producing IL-10 (224, 225). Gal-1 suppresses the degranulation of mast cell and, therefore, it exerts an anti-inflammatory role inhibiting the secretion of pro-inflammatory cytokines (129). In skin inflammatory diseases, it is observed that the treatment with rGal-1 reduces the infiltration of eosinophils and mast cells and the atopic dermatitis features (63). In relation to ACD, the role of Gal-1 expression in mast cells in the progression of this pathology is still unknown. Additional experiments are required to analyze whether Gal-1 in mast cells might have the same anti-inflammatory effect as in atopic dermatitis.

6.3. GAL-1 AND ENDOTHELIUM

During an inflammatory process, the first event is the leukocyte adhesion necessary to emigrate to the tissues (226). The lymphocyte recruitment into inflamed skin is mediated by different processes such as the binding to vascular endothelial cells and extravasation. Haptens increase the endothelial expression of adhesion molecules involved in the rolling, adhesion and extravasation of leukocyte to inflammation site such as E- and P-selectins, VCAM-1 and ICAM-1 (226-228). In particular, VCAM-1 and ICAM-1 are expressed in vascular endothelium in CHS model (229, 230) and their up-regulation takes place during the elicitation phase mediated by local release of TNFα (226). The interaction of LFA-1 and ICAM-1 is important to regulate T cell migration to inflamed tissues (231, 232). ICAM-1 blockade dampens not only T cell crawling, adhesion, and transmigration *in vitro*, but also reduces T cell migration from inflamed ears to the drained-lymph nodes in CHS model (233). However, our data show that neutralization of ICAM-1 does not prevent the enhanced inflammation mediated by the absence of Gal-1.

The expression of Gal-1 is increased on the endothelial cell surface by the *in vitro* activation with LPS or cytokines or by an inflammatory situation (234, 235). Gal-1 is highly expressed by vascular endothelial cells located in the inflamed site and can regulate the entrance of immune cell to the inflamed tissue. Human lymphatic endothelium also expresses Gal-1 and its deposition in extracellular matrix controls the migration of immunogenic human DCs (218). Moreover, *in vitro* experiments with endothelial cells treated with prostate cancer cell conditioned medium enriched in Gal-1 demonstrated that Gal-1 reduces not only the transendothelial migration of T cell but also their migration through the extracellular matrix leading to an anti-inflammatory role (235). On the other hand, Gal-1 does not alter the adhesion of T cells to the endothelium (235). However, it has not been ruled out that Gal-1 expression directly affects the migration of activated $CD4^+$ and $CD8^+$ T cells to lymph nodes or inflamed tissue in CHS model induced

by OXZ. Taking into account this experimental evidence, we could hypothesize that the absence of Gal-1 promotes the inflammation due to the increment of migration of the main players into the inflamed skin, what could be important in the development of CHS. However, our observations indicate that Gal-1 expressed by non-lymphoid cells such as endothelial cells does not participate in the modulation of T cell migration, since $CD4^+$ and $CD8^+$ T cells expressing Gal-1 are similarly recruited to the CHS site in Gal-$1^{-/-}$ and Gal-$1^{+/+}$ recipient mice.

6.4. GAL-1 AND T LYMPHOCYTES IN CHS

In the development of CD, both αβ T cells and γδ T cells play relevant roles (71, 236, 237). CHS model is a delayed type hypersensitivity reaction mediated by T cells that can recognize haptens loaded in MHC molecules (66). DETC and dermal γδ T cell subsets are involved in CHS response. Although the role of DETC in this model is controversial (39, 77, 78), depletion of Vγ4 T cells reduces the CHS response (39).

Gal-1 expression is modulated in different pathological situations such as inflammation, infection, autoimmunity, allergy, cancer, etc. Since Gal-1 is a member of a wide and diverse family, the analysis of the function of each member is difficult to evaluate with *in vivo* experiments because of their wide expression and overlapping roles and specificities. Thus, Gal-$1^{-/-}$ mice do not present major alterations probably thanks to the compensatory events (129, 223, 238-240). Most of the experiments to study the Gal-1 functions are carried out with the exogenous treatment of rGal-1 and demonstrate the relevant role of this lectin in the regulation of many biological processes such as cell growth, metastasis, cell-cell and cell-extracellular matrix interactions and immunomodulation (239, 241). However, the experiments with transgenic mice are commonly used to study the role of Gal-1 in different inflammatory pathologies (137, 242, 243). Gal-1 plays an important immunoregulatory role through its ability to modulate T-cell effector functions in different pathologies such as Crohn's disease (244), multiple sclerosis (245) or allergy (246). Moreover, Gal-1 is involved in tumor progression and tumor immune escape (125, 135, 247). Regarding inflammatory skin diseases, the administration of rGal-1 or mouse Gal-1 human chimera curbs inflammation (63, 126).

Although the two phases that conform the CHS model are relevant to establish this disorder (248), the role of Gal-1 in T lymphocyte-dependent inflammatory response to OXZ seems to be restricted to the elicitation phase. We found no evidence of a role for Gal-1 in the sensitization phase, as Gal-$1^{-/-}$ and Gal-$1^{+/+}$ mice developed the same magnitude of hapten-induced response.

Gal-1 is expressed in bovine γδ T cells (249). Moreover, γδ T cells can promote the tumor progression through the suppression of T cell responses by the secretion of Gal-1 (250). In addition, cancer-infiltrating $V\gamma9^+\gamma\delta$ T cells expressing Gal-1 are found in human ovarian tumors (251). Although there are many studied related to Gal-1 and γδ T cells in tumors, the role of Gal-1-expressing γδ T cells in contact hypersensitivity is unknown. Our data demonstrate that IL-17-secreting γδ T cells are increased in Gal-$1^{-/-}$ mice after the elicitation phase, indicating its potential role in the inflammatory response. However, dermal and epidermal γδ T cells are radioresistant populations in chimeric mice transplanted with bone marrow

cells from Gal-1$^{-/-}$ and Gal-1$^{+/+}$ mice (39), where the phenotype of full deficient mice is recapitulated. In addition, adoptive transfer experiments with purified CD8^{+} T cells are sufficient to generate increased inflammatory response in the absence of Gal-1. These data showed that the expression of Gal-1 on γδ T cells does not exert a key role in the development of CHS model, although they can contribute to the increased inflammatory response in Gal-1$^{-/-}$ mice.

On the other hand, it is commonly accepted that αβ T cells play a relevant role in ACD development. Many studies showed that Th17 and CD8^{+}IL-17^{+} are important in the elicitation phase of ACD disease (252-255). Memory CD8^{+} T cells are antigen-experienced cells that show an improved response to the second challenge, in comparison to naïve cells. The role of central memory CD8^{+} T cells and effector CD8^{+} T cells detected in the skin during inflammation has been established in CHS model as well as in viral infection (256-258).

Furthermore, the endogenous expression of Gal-1 in effector T cells and its role as a modulator of T cell functions has been described. Transcriptional analysis of different genes such as *Gata-3* and *Foxp3* showed that Gal-1 mainly promotes CD4^{+} T cells differentiation into Th2 and Treg linages upon activation with α-CD3 and α-CD28 (259). Thus, Gal-1 acts as a cross-regulatory molecule that limits Th1 survival and promotes Th2 cytokine (IL-4, IL-5 or IL-10) production (260). On the other hand, the expression of Gal-1 by Treg cells is increased after activation and the absence of this lectin dampens their regulatory function (133). Besides, Gal-1 activates the development of type 1 regulatory Th cells (Tr1) that present immunosuppressive capacity due to the production of IL-10 (135). Previous studies in our laboratory have shown that the interaction between Gal-1 and CD69 modulates Th17 effector cell differentiation and function (207). Additionally, Gal-1 inhibits cell-growth and promotes the apoptosis of activated immune cells (130-132), for example, the proliferation, survival and function of CD8^{+} T lymphocytes are downregulated by this lectin (261). Indeed, collagen-induced arthritis clinical manifestations dampen with the rGal-1 treatment due to the apoptotic features of Gal-1 (239). In contrast, the role of Gal-1 in the establishment of memory T cells is still unknown.

In relation to inflammatory skin disorders, the treatment with rGal-1 reduces the typical features of atopic dermatitis decreasing the skin thickness, the leukocyte recruitment from the blood and plasma IL-17 levels (63). In CHS, the administration of mouse Gal-1 human Ig chimera promotes a decrease in ear swelling and leukocyte infiltrate (126). Moreover, DCs that express transgenic Gal-1 induce apoptosis of activated T cells and dampen the elicitation phase of CHS model (220). Although these studies have described the relationship between Gal-1 and skin inflammation mediated by T cells, it is unknown whether Gal-1 from the non-lymphoid compartment contributes to CHS development, and whether endogenous Gal-1 directly influences on T cell subpopulations. Our data from chimeric mice experiments using Gal-1$^{+/+}$ recipient mice ruled out the potential effect of circulating soluble Gal-1 protein in the mechanism of control of skin inflammation. Our results show that the proliferation of CD8^{+} T cells in skin is similar in Gal-1$^{+/+}$ and Gal-1$^{-/-}$ in presence or absence of CD4^{+} T cells in OXZ-mediated CHS model. In addition, we demonstrate that the expression of Gal-1 controls the generation of central memory CD8^{+} T cells, and the

secretion of IFNγ, which account for the exacerbated skin inflammation. This role of Gal-1 in the generation of memory is a novel effect and requires additional studies to completely understand the mechanism. Hence, this work highlights the protective role of endogenous Gal-1 expression in $CD8^+$ T lymphocytes in the control of CHS-induced skin inflammation.

6.5. LAT1 AND NON-LYMPHOID CELLS

There are several studies related with metabolism and amino acid transporters in non-lymphoid cells due to their relevance in the control of cell function (146). In psoriasis there is an aberrant interaction of immune cells and KCs that maintains the characterized inflammatory and immune response in this pathology (262). In relation with the metabolism of KCs, it is described that the mTOR pathway is hyperactivated in KCs from patients with psoriasis (263). mTOR is a nutrient sensor and it can be activated by a huge amount of nutrients (179). Its aberrant induction is mediated by pro-inflammatory cytokines, such as IL-1β, IL-17A, and TNFα, increasing proliferation and reducing expression of differentiation markers (264). Our results indicate that, although LAT1 is induced in KCs in human psoriatic skin and in mouse skin after IMQ treatment, its expression is not essential to control of KC proliferation, indicating a functional compensation with alternative amino acid transporters, such as LAT2 or LAT3, which are also detected in the epidermal layer.

Neutrophils are the most abundant innate immune cells and in pathologic situations such as psoriasis their number increases as we demonstrated in this work. In fact, this large increase in neutrophils is considered as a characteristic feature of this inflammatory skin disease. In addition, the main functions of these cells also increase, which are related with the progression of psoriasis. These functions involve respiratory burst with the production of reactive oxygen species (ROS), degranulation and formation of neutrophil extracellular traps (NETs), playing a relevant role in the pathophysiology of psoriasis (265).

RNAseq analyses from human blood neutrophils have demonstrated the expression and regulation of different transporters in this population. Among the SLC members detected, Slc7a5 is one of them and is expressed in resting human blood neutrophils and overexpressed in the presence of TNFα, a regulator of neutrophil functions (266). *In vivo* and *ex vivo* experiments demonstrated that L-amino acids alter the activity of human peripheral neutrophils. All amino acids, but arginine, promote the inhibition of ROS release by neutrophils leading to the inhibition of the neutrophil stimulation (267). On the other hand, in colitis mouse model, it has been reported that CD98 is upregulated in inflammatory condition and the treatment with anti-CD98 antibody as an agonist induces inflammatory cell infiltration such as neutrophils (268). Although the relevance of neutrophils in psoriasis is well described, the functional role of CD98/LAT1 in these immune cells in the context of psoriasis is unknown and additional research would be required to elucidate this issue. However, taking into account the previous experimental evidence, the overexpression of LAT1/CD98 in activated neutrophils may aggravate skin inflammation as in other inflammatory disorders such as colitis.

The role of LAT1 as an essential amino acid transporter in activated T cells is more extensively studied than the relevance of this transporter in monocytes and macrophages. These myeloid cells are

important players in the progress of inflammation and participate in the pathogenesis of several inflammatory disorders. It is described that SLC7A5 is overexpressed in activated human monocytes and macrophages and can regulate their metabolic processes. For instance, LAT1 mediates leucine intake that promotes IL-1β production in activated monocytes and macrophages through the increase of mTORC1 activity and glycolysis (269). LAT1 expression studies in circulating monocytes from rheumatoid arthritis patients demonstrated that amino acid influx through this transporter modulates inflammatory conditions (269). Moreover, it has been demonstrated that monocytes can be differentiated into two types of macrophages, M1 that have "pro-inflammatory" properties and M2 macrophages, which are "pro-resolving". In the first subpopulation the SLC7A5 expression is upregulated, but the expression levels of SLC7A8 (LAT2) are similar in both types after the activation with lipopolysaccharide (LPS) (269). Therefore, the inhibition of LAT1 in macrophages could be a potential therapy to treat patients with different inflammatory disorders such as psoriasis.

On the other hand, DCs cells are essential in the initiation of the immune response (270, 271). DC metabolism regulation and control of mTOR activation are important in the differentiation and immune functions of these cells. For example, mTORC1 is crucial for the conservation of LC in the skin of mice (272, 273). Considering the role of DCs in the physiopathology of inflammatory diseases, DCs firstly sense the environment and they are activated by the interaction of different molecules with the TLR receptor. TLR ligands activate mTORC1 and mTORC2 and maintain the production of pro-inflammatory signals. Then, DCs migrate to lymph nodes where they can activate T cells, although it is known that mTOR limits their capacity in this phase. The dual role of mTOR in DC responses depends on the nutrient, energy and oxygen content in the surrounding environment. For instance, mTOR can sense a huge amount of different nutrients in the periphery and promote their activation and a glycolytic response. In contrast, in the lymph nodes, activated T cells compete with DCs for the nutrients and this environment with restrictions dampens mTOR in DCs. Therefore, inhibition of mTOR in the periphery promotes anti-inflammatory effect, but in the lymph nodes, this inhibition increases T cell activation (273, 274).

In relation to CD98/LAT1 complex, it has been described that CD98 is expressed on DC surface and plays a crucial role in T cell activation induced by DCs providing a co-stimulatory signal during APC-T cell presentation (275). However, the role of both components in DCs in the progression of psoriasis is still unknown. Considering these observations, the inhibition of LAT1 in DCs could act as a possible target to treat psoriasis.

Mast cells are considered as effector cells in allergy but also these cells can participate in innate and acquired immunity and in inflammation processes (225). Human mast cells can interact with T cells leading to their reciprocally activation through the expression of adhesion molecules such as ICAM-1. Also, mast cells express CD80 and CD86 that can activate T cells. The activation of mast cells promotes their degranulation, migration and adhesion to extracellular matrix and endothelial cell ligands (225, 276). As it is mentioned before, these cells express IL-10, and thus, they can exert an immunosuppressive function. The number of mast cells increases in human psoriatic lesion and they expresses IFNγ, IL-22 and IL-17

(225, 277, 278). Moreover, microarray analyses of mast cell from different mouse organs show that Slc7a5 is expressed in these cells (279). Even though mast cells are increased in psoriasis lesions and these cells express LAT1, it is unknown the role of the inhibition of this transporter in these cells in the progression of this pathology.

6.6. LAT1 AND T LYMPHOCYTES IN PSORIASIS

Numerous disorders are caused by an aberrant function of immune cells. Immune cells during their life require diverse quantities of energy and nutrients. Several metabolic pathways are activated or suppressed to control different immune cell processes such as differentiation, proliferation, development or activation (146, 280). During immune response, T cells recognize an antigen leading to cytokine production and cell growth. These processes require huge energy levels and intracellular biosynthesis and therefore, T cell activation needs a metabolic switch. Apart from glucose, T cells demand amino acid incorporation to maintain the increased requirements of protein synthesis (179, 180). Although in activated human T cells LAT1 is the major transporter for essential amino acids, its expression is very low in normal cells, but its functionality is necessary to the cellular activity in both situations. Moreover, because LAT1 is overexpressed in several types of cancers, many studies have addressed the inhibition of this transporter as a potential therapeutic treatment. The JPH203 pharmacologic compound inhibits leucine uptake promoting a reduction in cytokine production by activated human T cells (179). Hence, LAT1 inhibition dampens the growth of different cancer cell lines and *in vivo* experiments show that JPH203 decreases tumor growth in xenograft models of human leukemia and colon cancer cells (281-283).

In relation to skin inflammatory disorders, LAT1 expression is increased in atopic dermatitis and its inhibition by JPH203 attenuates Th2 activation and, therefore, allergic inflammation (284). Regarding psoriasis, SLC7A5 expression was detected in skin samples of psoriatic patients (162). A major feature of the IMQ model is the expansion of $CD27^{-}\gamma4^{+}\delta4^{+}$ T cells in dLNs that produce IL-17 (96, 121, 171). A similar expansion of this population is observed in a mouse experimental autoimmune encephalomyelitis model (285). Although the increased frequency of γδ T cells has also been observed in human psoriatic lesions (97), their relevance in the onset or recurrence of this disease is not clearly established (196, 197). Previous studies in our laboratory demonstrated that CD69 controls the uptake of L-Trp through LAT1 promoting the secretion of IL-22 induced by AHR in γδ T cell (162). Additionally, LAT1 inhibition controls the proliferation of $V\gamma4^{+}\delta4^{+}$ T cells, even in the absence of AHR. $IL\text{-}17^{+}\gamma4^{+}\delta4^{+}$ T-cell expansion after IMQ treatment is blocked by targeting LAT1 pharmacologically or genetically. Importantly, LAT1 inhibition also blocks the expansion of human γδ T cells. LAT1 deletion in dermal γδ T cells also effectively controls IL-23-induced skin inflammation. Further experiments will address whether LAT1 deletion controls dermal γδ T-cell expansion in patients with other IL-17-mediated diseases, such as autoimmune encephalomyelitis. In depth analyses of skin human γδ T cells detected in homeostasis and in different diseases will undoubtedly improve novel therapeutic strategies.

mTOR can sense nutrient availability and its activation by several nutrients induces protein synthesis and cell cycle progression and inhibits autophagy (179). General amino acid control nonderepressible 2

(GCN2) is activated by deprivation, and the induced signaling cascade regulates the expression of different genes that are relevant in stress response (286). Furthermore, PI3K/AKT/mTOR axis is relevant for T cell metabolism and differentiation and for the inhibition of Treg generation (180). In addition, IL-1β induces mTOR activation that synergizes with IL-23 to promote dermal γδ T cell proliferation and IL-17 production, and hence they increase skin inflammation. Both mTORC1 and mTORC2 are important to dermal γδ T cells but exclusively the absence of mTORC2 dampens this population (287). The capacity of amino acids to regulate mTOR pathway is conserved across eukaryotes (288). Our data indicate that inhibition of PI3K/AKT or amino acid depletion abrogates mTOR activation induced by IL-23 and IL-1β in γδ T cells. Importantly, PI3K/AKT signaling is important for the expansion of IL-17-secreting γδ T cells (289, 290). Knowledge of the molecular mechanisms that define the function of γδ T cells in skin homeostasis and skin diseases will identify novel strategies to improve the resolution of inflammation.

In relation to αβ T cells, which are the main players in the human psoriasis (176), our data demonstrate that LAT1 deletion in $CD4^+$ T cells dampens IMQ-induced skin inflammation. Moreover, LAT1 inhibition successfully impairs the Th17 differentiation program in human $CD4^+$ T cells. Whether mTOR activation is required for Th17 cell expansion has been studied extensively (291); however, the role of Th17 driver cytokines, such as IL-23 and IL-1β, in mTOR activation had not been fully characterized. Th17 cell development requires TCR-mediated activation, which triggers the PI3K/AKT/mTOR signaling axis. LAT1 expression is induced after TCR engagement and is essential to properly activate αβ T cells (154). LAT1 expression after TCR activation requires NF-κB and activator protein 1 activation (179). Our data clearly show that IL-23 and IL-1β stimulation also regulates LAT1 expression in Th17 cells, as well as in γδ T cells, which do not receive TCR input. IL-23-induced Janus kinase 2 activation triggers the PI3K/AKT and NF-κB pathways (292). IL-1β stimulation can induce NF-κβ activation through myeloid differentiation response gene-88 (293), which is also essential for mTOR activation in Th17 cells (294). Moreover, IL-1R signaling induces PI3K/AKT phosphorylation and mTOR activation to promote differentiation of pathogenic Th17 cells (295). Our data suggest that IL-23 and IL-1β induce LAT1 expression as a positive feedback loop to drive activation of PI3K/AKT/mTOR pathway, which is essential for the increased survival and expansion of IL-17-releasing cells. LAT1 inhibition in the presence of rapamycin, the canonical mTOR inhibitor, had no additive effect. Inhibition of mTOR by targeting LAT1 could be an alternative approach to specifically target immune cells stimulated by IL-23 and IL-1β in patients with inflammatory diseases. Furthermore, LAT1 inhibition decreases transcriptional levels of IL-1β in the skin. This effect is potentially related to the blockade of amino acid uptake in skin macrophages, which is also involved in IL-1β secretion (269). This additional anti-inflammatory effect of JPH203 might contribute to the prevention of psoriasis. However, genetic deletion of LAT1 in lymphocytes, including RORγt-expressing cells, such as γδ T cells and Th17 cells was sufficient to control disease development.

6.7. FUTURE PERSPECTIVES: LAT-1, GAL-1, ACD AND PSORIASIS

Because ACD and psoriasis are prevalent and complex inflammatory skin disorders (296, 297), extensive studies about cellular and molecular mechanisms involved in these pathologies are necessary to improve the diagnosis and to develop novel therapies. During the last years, there are many studies about

the pathogenesis of ACD and psoriasis. Comparing both disorders there are differences not only at clinical manifestation level but also at cellular level. For example, psoriasis is characterized by KC hyper-proliferation and apoptosis resistance but the pathogenic hallmark of ACD is the induction of KC apoptosis by T cells (298, 299). Genome expression analysis shows that pathways related with metabolism and proliferation are altered in clinically non-involved skin of psoriasis patients with respect to non-psoriatic patients. Therefore, both pathologies are different immune reactions and their mechanisms are independent and do not interfere between them (183).

In regard to the ACD treatment, the diagnosis and the recognition of the causing agent is critical. Due to the clinical similarities with other skin pathologies such as atopic dermatitis, ICD, seborrheic dermatitis, etc., it is difficult to distinguish them. Besides, some of these pathologies may coexist in the patient, what can make the diagnosis more difficult. To perform the diagnosis, the use of clinical history, patch testing and biopsies is very frequent. Although the complete avoidance of the offending allergen is fundamental in the remission, antihistamines and topical corticosteroids may be necessary. In severe manifestations of ACD, systemic steroids and phototherapy (e.g. UVB) are typically used (65, 300). Due to the current limitations in the ACD treatment, novel therapies that specifically target molecules of relevant pathways or cells involved in the progression of the disease are necessary. Taking into consideration the critical molecules in the development of CHS, the IL-4 receptor α inhibitor (Dupilumab) might be effective in the inhibition of Th2-mediated ACD induced by weak allergens such as fragrances (301).

The relation between commensal microbiota and immune system is well known (302, 303). Some studies demonstrate that certain food component (304, 305), probiotic *Lactobacillus casei* (306) and non-digestive oligosaccharides such as fructo-oligosaccharide (FOS) reduce CHS in mice. For example, the protective role of FOS is associated with changes in their intestinal microbiota leading to an increase in *Bifidobacterium pseudolongum* (194).

Pharmacological interventions with rGal-1 protein plays an essential immunoregulatory role by inhibiting $CD4^+$ T-cell effector functions in different pathologies such as Crohn's disease (244), multiple sclerosis (245) or asthma (246). Regarding inflammatory skin diseases, the administration of rGal-1 has been proved to be effective in the control of inflammation (63, 126). However, it is unknown whether endogenous expression of Gal-1 directly influences T cell subpopulations function, and whether Gal-1 from the non-lymphoid compartment contributes to CHS. Our work shows that endogenous Gal-1 expression in the T lymphocytes is sufficient to control the inflammation in the CHS model. In conclusion, this work highlights the protective role of cellular expression of Gal-1 in effector $CD8^+$ T cells but not in $CD4^+$ T cells in the development of this cutaneous inflammatory disease. The development of central memory $CD8^+$ T cells as well as the secretion of IFNγ by effector $CD8^+$ T cells are increased in the absence of Gal-1. In contrast, the regulatory capacity of Treg cells does not appear to be affected in CHS by the genetic deletion of Gal-1.

In relation to ACD and LAT1, is still unknown whether the inhibition of this transporter could play a protective role in this pathology. Taking into account the effect of LAT1 in other skin inflammatory

diseases such as atopic dermatitis (284), it is plausible that JPH203 may exert a protective role in the development and progression of ACD.

Regarding the psoriasis, there are different treatments depending on the injury spread (localized or diffused) and the presence of comorbidities. It is very frequent that psoriatic patients develop psoriatic arthritis, cardiovascular disease, kidney disorders, metabolic syndrome, malignancy, infection and mood disorders. Additionally, this pathology affects the quality of life, work productivity, physical and mental functioning. The possible therapies include topical, phototherapy, systemic and biological treatments (307-311). Patients with mild manifestations of the disease can be treated with topical treatments, which present high efficacy-to-safety ratio. Among the available topical therapies, vitamin D analogues and corticosteroids are the most used in psoriasis. Topical corticosteroids-treated patients show a significant improvement in symptoms in the first month of the therapy but also, develop adverse reactions and tachyphylaxis with the recurrent and continuous applications. For these reasons, to avoid side effects clinicians employ other topical options such as vitamin D analogues that can be used in combination with other therapies like corticosteroids or alone. Vitamin D3 analogues are well tolerated by patients although it is important to maintain the recommended doses because it can cause hypercalcemia. These analogues not only regulate the metabolism of calcium and phosphorus but also control cell growth, differentiation and immune functions (311, 312). For instance, vitamin D analogues control the hyperproliferation of KCs. Besides, calcitriol and others analogues show immunomodulated functions on monocytes, macrophages, DCs, and T cells (313). Moreover, vitamin D3 inhibits the production of different cytokines such as IL-2, IL-6 or IFNγ (312). Clinical trials studies demonstrate the efficacy and safety of calcitriol **(Table A.1)** (312, 314-316).

When patients do not respond to topical treatment or present extensive psoriatic lesions, it is employed narrowband UVB (311-312 nm) or psoralen plus ultraviolet A (PUVA) that consists in the administration of a photosensitizing psoralen and the exposition to 320-400 nm UV light. Although both therapies are effective in psoriasis, they are highly mutagenic and promote long-term risks such as skin cancer (92, 311). Furthermore, systemic treatments such as methotrexate, oral retinoids and cyclosporine are relevant in the treatment of severe psoriasis. Patients treated with methotrexate show great improvement but many side effects such as myelosuppression, hepatic fibrosis and pulmonary fibrosis. Moreover, cyclosporine is an effective treatment but also increases the risk of infection and non-melanoma skin cancer. Due to its cumulative toxicity, it is suitable for short-term treatment. On the other hand, acitretin is the unique oral retinoid accepted for psoriasis treatment. It is less effective than the other systemic therapies but only few percentage of treated psoriatic patients show side effects **(Table A.1)** (311).

The increasing knowledge of the immunopathogenesis of psoriasis has led to the appearance of potential novel therapies based on the modulation of the immune response at a cellular or molecular level. In relation to the cellular level, alefacept (LFA-3 fused to IgG) was the first biological therapy used for psoriasis and can bind to CD2 on activated memory T cells promoting their apoptosis. Additionally, efalizumab is a CD11a monoclonal antibody that inhibits the interaction between LFA1 and ICAM-1 and

therefore the T cell extravasation and immunological synapse. They were the first generation of immune-targeted biologic drugs approved in 2003. Moreover, the knowledge of the molecular mechanisms involved in the progression of this pathology has led to the development of new therapies that directly target cytokines or receptors critical in the development of this disorder. For instance, TNFα antagonists (adalimumab, etanercept, infliximab and certolizumab pegol), which decrease the clinical manifestations of psoriasis, are used as monotherapies or in combinations with other treatment **(Table A.1)** (99, 311). In addition, other monoclonal antibodies are approved by the U.S. Food and Drug Administration: ustekinumab (IL-12/IL-23 inhibitor); secukinumab and ixekizumab (IL-17A inhibitors); brodalumab (IL-17RA inhibitor); and guselkumab, tildrakizumab and risankizumab (IL-23 inhibitors) (99) **(Table A.1)**. Clinical trials studies demonstrated that monoclonal antibodies against IL-12, IL-23 and IL-17 show efficacy to reduce the typical manifestations in psoriatic patients, and present favourable side effect profiles in comparison to TNFα antagonists (99, 307). In Europe, these immunobiological therapies are only allowed in case of severe manifestations of the disease when the other treatments do not promote improvement in the patients (92, 308, 311, 317). Due to the complexity of the cellular and molecular mechanisms involved in the progression of psoriasis, it is important to increase our knowledge in order to generate novel drugs to treat and improve the quality of life of psoriatic patients. As mentioned above, the side effects observed in the patients treated with the available therapies are very diverse and many of them cause serious clinical damages. More studies are needed to improve the overall health of patients with effective treatments that can shorten the long-term therapies and the large costs associated to them.

Several studies describe the different microbiota in psoriatic patients with respect to healthy people. However, the direct relation between the skin microbiota and the pathogenesis of psoriasis is not clear enough. The commensal microbiota exerts a relevant role in the maintenance and functionality of skin barrier. In addition, the intestinal microbiota has been related with skin disorders (318, 319). Among the skin microbiota, the most representative phyla are Firmicutes and Proteobacteria, while Atinobacteria is the less abundant in psoriatic lesions (320). Additionally, in these lesions there is an increase in the concentration of *Malassezia* spp. *Malassezia furfur* can induce KC hyperproliferation and the migration of immune cells via the upregulation of tumour growth factor-β1, integrin chain and HSP70 expression (319, 321). Some authors speculate that the efficacy of conventional therapies such as narrowband UVB may be modulated by alteration in skin microbiota. It is described that this phototherapy not only promotes an improvement in oxidative stress parameters and thus in the pathology, but also, modifies the skin microbiota. More studies are necessary to confirm whether there is a direct relationship between these components (319, 322, 323). A case report about a patient with pustular psoriasis showed that the probiotics treatment (*Lactobacillus*) improves the psoriatic lesions (324). The psoriasis features induced by IMQ dampen in mice feed with *Lactobacillus pentosus* in comparison to control mice (325). Topical treatments such as vitamin D analogues modify AMP expression. For instance, calcipotriol increases the expression of cathelicidin, which acts against the fungus leading to an alteration in the *Malassezia* population. More studies are necessary to demonstrate whether the classical treatments in combination with the microbiota could be a good option to obtain better results in the psoriasis progression. Moreover, another possible

therapeutic target for psoriasis could be the restoration of symbiosis by selective modulation of skin microbiota (319, 326).

On the other hand, studies of the cellular mechanisms that participate in the progression of psoriasis demonstrated the relevant role of resident memory T cells (TRMs) in the reactivation and recurrence of skin lesions. Thus, the inhibition of the long-term survival of TRMs in skin could be a potential therapy (108, 176). Moreover, neutrophils functions such as degranulation, NETs formation and ROS generation are crucial in the immunopathogenesis of psoriasis, and thus their inhibition could be possible targets to treat this pathology (265). Other study demonstrates that the inhibition of RORγt in mice through an inverse agonist promotes a decrease in Th17 cell differentiation and in IL-17A production by Th17 and γδ T cells leading a reduction in IMQ-induced psoriasis features (327).

Furthermore, many studies analyzed the main role of mTOR in psoriasis. The topical application of rapamycin reduces the characteristic features of psoriasis, i.e. erythema, scaling, thickness and neovascularization, in IMQ-treated skin (328, 329). The same protective effect is observed in patients systemically- or topically- treated with rapamycin (sirolimus) (330, 331). In addition to the inhibition of mTOR, our work provides the first evidence that murine psoriasis models can be precisely modulated by targeting inflammatory cell metabolism. Our data postulate that the LAT1 inhibitor JPH203, which has already been tested in patients with cancer for biosafety (159, 332), could be an alternative therapy to control chronic skin inflammation. Therefore, we propose that blocking LAT1-mediated amino acid transporter merits consideration as a novel strategy to control the expansion of innate and adaptive T cells in the reactive state in patients with inflammatory diseases. This inhibitor could be an alternative and effective treatment that could reduce the high costs generated by other therapies due to the long-term treatments. More studies are needed to find new targets that could generate potential therapies with long-term safety and tolerability and also, that they could promote long-lasting remissions. The effect of Gal-1 could also be interesting to study in psoriasis. In this study, we demonstrated its protective role in ACD but it is still unknown whether this galectin may be used as an anti-inflammatory drug or its inhibition could be required to dampen the psoriasis development.

In addition, more studies related to metabolomic and proteomic are necessary to underscore new potential targets to treat both pathologies, ACD and psoriasis. To further understand the mechanism mediating γδ T-cell functions, novel genetic strategies specifically targeting different subsets of skin γδ T cells, as well as the molecules involved in antigen recognition, need to be explored in murine models of psoriasis and dermatitis. Additional research about the molecular mechanisms that define the function of γδ T cells and the establishment of memory T cells in psoriasis and ACD is necessary to discover novel strategies to dampen skin inflammation. Besides, more studies with human samples are required to analyze the expression of LAT1 and Gal-1 and further improve the knowledge of the mechanisms involved in these disorders to develop novel, effective and safe therapies.

6.8. CONCLUDING REMARKS

This thesis work reveals new potential targets in the development and progression of different prevalent inflammatory skin diseases such as ACD and psoriasis. The role of both players, Gal-1 and LAT1, seems to be relevant in ACD and psoriasis, respectively. Both, recombinant Gal-1 and the pharmacologic inhibitor JPH203, could be used as anti-inflammatory drugs in ACD and psoriasis, respectively, because of their protective effects in the development of these inflammatory skin disorders **(Figure 6.1)**.

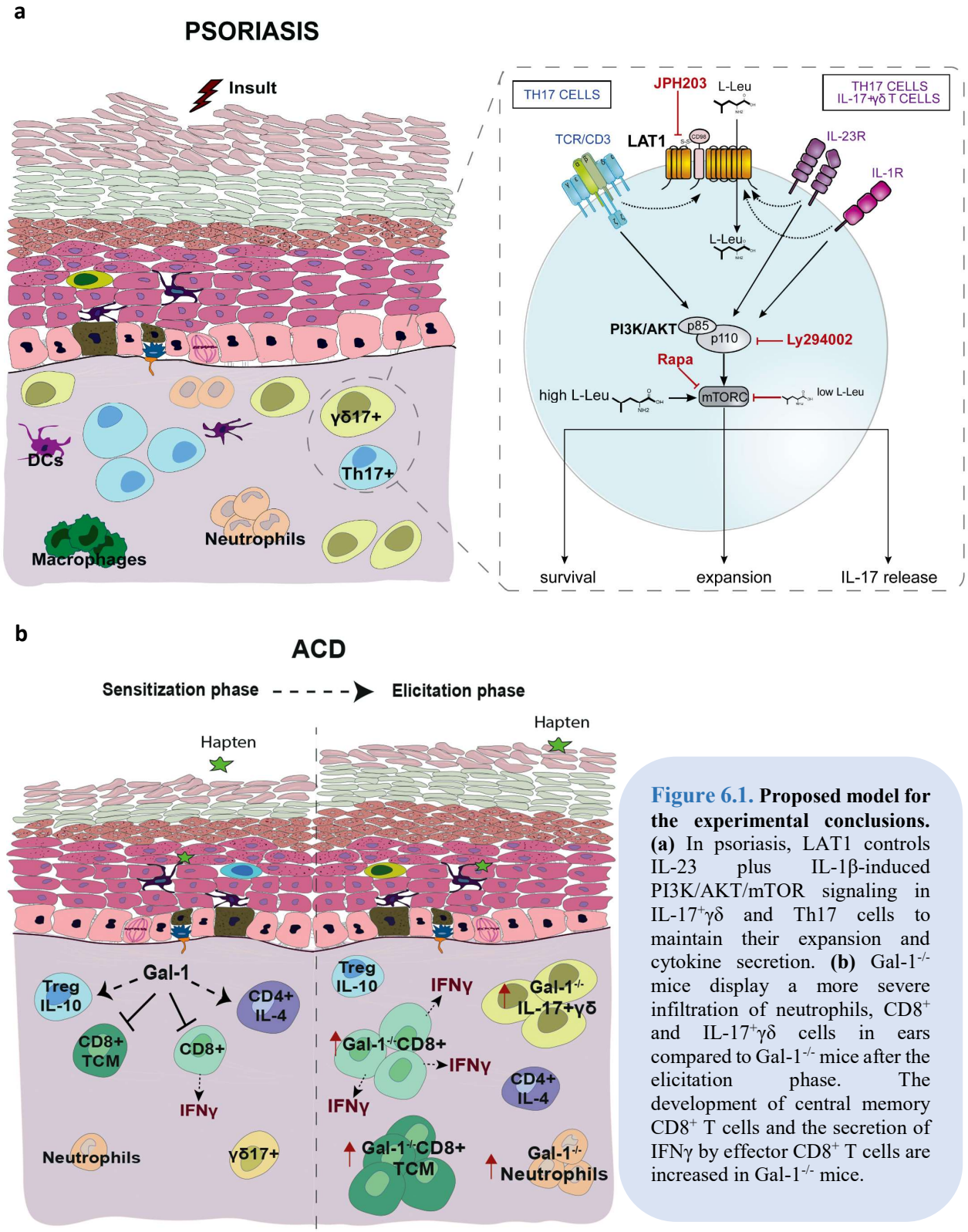

Figure 6.1. Proposed model for the experimental conclusions. (a) In psoriasis, LAT1 controls IL-23 plus IL-1β-induced PI3K/AKT/mTOR signaling in IL-17^{+}γδ and Th17 cells to maintain their expansion and cytokine secretion. **(b)** Gal-1$^{-/-}$ mice display a more severe infiltration of neutrophils, CD8^{+} and IL-17^{+}γδ cells in ears compared to Gal-1$^{-/-}$ mice after the elicitation phase. The development of central memory CD8^{+} T cells and the secretion of IFNγ by effector CD8^{+} T cells are increased in Gal-1$^{-/-}$ mice.

CONCLUSIONS

7. CONCLUSIONS

Our findings showed here support the following conclusions:

1. Gal-1 controls the inflammatory response in CHS model regulating the infiltration of neutrophils, $CD8^+$ and IL-17-secreting γδ T cells in ears after the elicitation phase.

2. Expression of Gal-1 in $CD8^+$ but not in $CD4^+$ T lymphocytes exerts a protective effect in the control of CHS-induced skin inflammation.

3. Gal-1 controls the development of central memory $CD8^+$ T cells as well as the secretion of IFNγ by effector $CD8^+$ T cells. In contrast, the regulatory capacity of Treg cells is not affected in CHS by the genetic deletion of Gal-1.

4. LAT1 (SLC7A5) expression is increased in patients with psoriasis and in IMQ-treated mice in both keratinocytes and dermal infiltrating lymphocytes. In addition, LAT1 expression is upregulated by IL-23 and IL-1β in γδ and $CD4^+$ T cells.

5. LAT1 inhibition and/or deletion do not affect keratinocyte proliferation but impair expansion of IL-17-secreting γδ and $CD4^+$ T cells, and reduce inflammation in psoriasis-mouse models.

6. LAT1 controls IL-23 plus IL-1β-induced PI3K/AKT/mTOR signaling in IL-17-secreting γδ and $CD4^+$ T cells to guarantee their expansion and cytokine secretion.

CONCLUSIONES

8. CONCLUSIONES

Los resultados presentados en este trabajo permiten concluir:

1. Gal-1 controla la respuesta inflamatoria en el modelo de hipersensibilidad por contacto ya que regula la infiltración severa de neutrófilos, linfocitos $CD8^+$ y células T γδ productoras de IL-17.

2. La expresión de Gal-1 en las células $CD8^+$, pero no en las $CD4^+$, ejerce un papel protector en el control de la inflamación inducida en el modelo de hipersensibilidad por contacto.

3. Gal-1 controla tanto el desarrollo de células T de memoria central $CD8^+$ como la secreción de IFNγ por parte de los linfocitos efectores $CD8^+$. Sin embargo, la capacidad reguladora de las células Treg no está afectada por la deleción genética de Gal-1 en este modelo de hipersensibilidad por contacto.

4. La expresión de LAT1 está incrementada en queratinocitos y linfocitos infiltrados en lesiones psoriásicas de pacientes y en el modelo de ratón con IMQ. Además, su expresión en linfocitos T $CD4^+$ y γδ está inducida por las citoquinas IL-23 e IL-1β.

5. La ausencia de LAT1 en queratinocitos no afecta a su proliferación, pero sí disminuye la expansión de las células T γδ productoras de IL-17 y de los linfocitos T $CD4^+$ y el grado de inflamación psoriásica en modelos animales.

6. LAT1 controla la activación de la ruta PI3K/AKT/mTOR inducida por IL-23 e IL-1β en linfocitos T $CD4^+$ y γδ productores de IL-17, facilitando su expansión y la secreción de IL-17.

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ANNEXES

10. ANNEXES

Table A.1. Therapies used in psoriasis. Modified from (99).

Type	Treatments	
Mild Psoriasis	**Topical corticosteroids** (Anti-inflammatory, anti-proliferative, and locally vasoconstrictive effects via downregulation of genes coding pro-inflammatory cytokines)	- **Class I-VII**
	Topical Calcineurin inhibitors (Block T cell activationby unhibiting IL-2 and IFNγ synthesis)	- **Tacrolimus** - **Pimecrolimus**
	Topical Vitamina D analogues (Bind to vitamin D receptors on T cells and keratinocytes to block keratinocyte proliferation and boost keratinocyte diferentiation)	- **Calcitriol** - **Combination calcipotriene/calcipotriol**
	Topical keratolytics (Inhibit the proliferation of keratinocytes and/or reduce scaling)	- **Tazarotene** - **Salicylic acid**
	Targeted Phototherapy (To treat localized plaque psoriasis)	- **UV-B** (308 nm)
Severe Psoriasis	**Phototherapy** (Decreases DNA synthesis)	- **UV-B** (308 nm) - **PUVA:** psorales + UV-A (320-400 nm)
	Oral systemics (Downregulate cytokine production, dampen T cell activities, etc.)	- **Methotrexate** - **Apremilast** - **Acitretin** - **Cyclosporine** - **Fumarates**
	Biologics (Decrease the inflammatory cascade involved in psoriasis pathogenesis)	- **TNFα inhibitors** ○ Etanercept ○ Adalimumab ○ Infliximab ○ Certolizumab pegol
		- **IL-12/IL-23 inhibitor** ○ Ustekinumab
		- **IL-17 inhibitor** ○ Secukinumab ○ Ixekizumab ○ Brodalumab
		- **IL-23 inhibitor** ○ Guselkumab ○ Tildrakizumab ○ Risankizumab

Table A.2. The main gene families of amino acid transporters expressed in mammalian cells. Taken from (143).

Gene family	Acronym	Full name	AA substrates	Na^+-symport*	Classical transport system(s) nomenclature
SLC1					
Excitatory AA transporters	EAAT1–5	Excitatory AA transporter	AAA	+ (H^+-symport; K^+-antiport)	X^-_{AG}
	ASCT1, 2	ASC transporter	SNAA	+ (AA antiport)	ASC
SLC6					
Neurotransmitter AA transporters	GAT1-3	GABA transporter	GABA	+ (+ Cl^- symport)	GABA
	GLYT1,2	Glycine transporter	Gly	+ (+ Cl^- symport)	Gly
	IMINO	Proline/betaine transporters	Pro	+ (+ Cl^- symport)	Pro/β
	$ATB^{0,+}$	$B^{0,+}$ AA transporter	NAA, CAA	+ (+ Cl^- symport)	$B^{0,+}$
	B^0AT1, 2		B^0 AA transporter		NAA
SLC7					
Glycoprotein-associated AA transporters (gpaAT/HAT)	LAT1, 2	System L1 transporter (4F2 heteromer)	LNAA	– (AA antiport)	L
	y^+LAT1, 2	System y^+L transporter (4F2 heteromer)	NAA, CAA	–/+† (AA antiport)	y^+L
	xCT	Cystine-glutamate exchanger (4F2 heteromer)	Cys, Glu	– (AA antiport)	x^-_C
	ascT	System asc transporter (4F2 heteromer)	SNAA	– (AA antiport)	asc
	$b^{0,+}AT$	$b^{0,+}$ transporter (rBAT heteromer)	NAA, CAA	– (AA antiport)	$b^{0,+}$
Cationic AA transporters	CAT1–4	Cationic AA transporter 1, 2A/B, 3	CAA	–	y^+
SLC16					
Monocarboxylate transporters	TAT1	System T transporter	Aromatic AA	–	T
SLC36					
Proton-coupled AA transporters	PAT1–4	Proton-coupled AA transporters 1-4	Pro, Gly, Ala	– (H^+-symport)	PAT
SLC38					
Small neutral AA transporters (VGAT)	SNAT3, 5	System N transporter	Gln, Asn, His	+ (H^+-antiport)	N
	SNAT1,2,4	System A transporter	SNAA	+	A
SLC43					
Large neutral AA transporters	LAT3,4	System L2 transporter	LNAA	–	L

See, for example [2–4,5▪▪] for original sources and more detailed information about Classical Transport System nomenclature. The gpaAT are SLC7 subfamily members requiring an 'accessory' SLC3 subunit, either SLC3A1 (rBAT/NBAT) or SLC3A2 (F42hc/CD98) [3].
AA movements are uniport unless specified. * Na^+-symport designated +. † - Transport of NAA is Na^+-dependent and CAA is Na^+-independent.
AAA, anionic AA. (L/S)NAA, (large/small) neutral AA. CAA, cationic AA.

❖ Publications related with this work:

- **Castillo-González, R.***, Cibrian, D.*, Fernández-Gallego, N., Ramírez-Huesca, M., Saiz, M. L., Navarro, M. N., Fresno, M., de la Fuente, H. & Sanchez-Madrid, F. (2020). Galectin-1 expression in CD8+ T lymphocytes controls inflammation in contact hypersensitivity. *Journal of Investigative Dermatology*. doi: 10.1016/j.jid.2020.10.020. Online ahead of print.

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- Cibrian, D.*, **Castillo-González, R.***, Fernández-Gallego, N., de la Fuente, H., Jorge, I., Saiz, M. L., . . . Sanchez-Madrid, F. (2020). Targeting L-type amino acid transporter 1 in innate and adaptive T cells efficiently controls skin inflammation. J*ournal of Allergy and Clinical Immunology,* 145(1), 199-214 e111. doi:10.1016/j.jaci.2019.09.025

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- **Castillo-González, R.***, Cibrian, D.* & Sanchez-Madrid, F. (2020) Dissecting the complexity of γδ T cell subsets in skin homeostasis, inflammation and malignancy. J*ournal of Allergy and Clinical Immunology*. doi: 10.1016/j.jaci.2020.11.023. Online ahead of print.

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❖ Other articles not related with this work:

- Alvarez-Salamero, C.*, **Castillo-Gonzalez, R.***, Pastor-Fernandez, G., Mariblanca, I. R., Pino, J., Cibrian, D., & Navarro, M. N. (2020). IL-23 signaling regulation of pro-inflammatory T-cell migration uncovered by phosphoproteomics. *PLoS Biology,* 18(3), e3000646. doi:10.1371/journal.pbio.3000646

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- Labrousse-Arias, D., **Castillo-Gonzalez, R.**, Rogers, N. M., Torres-Capelli, M., Barreira, B., Aragones, J., ... & Calzada, M. J. (2016). HIF-2α-mediated induction of pulmonary thrombospondin-1 contributes to hypoxia-driven vascular remodelling and vasoconstriction. *Cardiovascular research*, 109(1), 115-130.

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❖ Other reviews not related with this work:

- Alvarez-Salamero, C.*, **Castillo-Gonzalez, R.***, & Navarro, M. N. (2017). Lighting Up T Lymphocyte Signaling with Quantitative Phosphoproteomics. *Frontiers in Immunology,* 8, 938. doi:10.3389/fimmu.2017.00938

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Galectin-1 Expression in CD8$^+$ T Lymphocytes Controls Inflammation in Contact Hypersensitivity

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Allergic contact dermatitis, also known as contact hypersensitivity, is a frequent T-cell–mediated inflammatory skin disease characterized by red, itchy, swollen, and cracked skin. It is caused by the direct contact with an allergen and/or irritant hapten. Galectin-1 (Gal-1) is a β-galactoside–binding lectin, which is highly expressed in several types of immune cells. The role of endogenous Gal-1 in contact hypersensitivity is not known. We found that Gal-1–deficient mice display more sustained and prolonged skin inflammation than wild-type mice after oxazolone treatment. Gal-1–deficient mice have increased CD8$^+$ T cells and neutrophilic infiltration in the skin. After the sensitization phase, Gal-1–depleted mice showed an increased frequency of central memory CD8$^+$ T cells and IFN-γ secretion by CD8$^+$ T cells. The absence of Gal-1 does not affect the migration of transferred CD4$^+$ and CD8$^+$ T cells from the blood to the lymph nodes or to the skin. The depletion of CD4$^+$ T lymphocytes as well as adoptive transfer experiments demonstrated that endogenous expression of Gal-1 on CD8$^+$ T lymphocytes exerts a major role in the control of contact hypersensitivity model. These data underscore the protective role of endogenous Gal-1 in CD8$^+$ but not CD4$^+$ T cells in the development of allergic contact dermatitis.

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INTRODUCTION

Contact dermatitis (CD) is a prevalent inflammatory skin disease affecting 15–20% of the general population. Two main different types of CD are (i) irritant CD, a primary skin reaction to chemicals, and (ii) allergic CD (ACD) or contact hypersensitivity (CHS), an immune reaction caused by contact with a hapten (Martin, 2013). A typical CHS reaction displays two phases: (i) sensitization, which occurs after the first contact with the hapten, and induces clonal expansion of specific central memory and effector T cells (Gaide et al., 2015) and (ii) elicitation, which is triggered by re-exposure to the hapten. This second phase comprises the reactivation and recruitment of specific central memory and effector CD4$^+$ and CD8$^+$ T cells at the site of allergen exposure (Allen, 2013; Saint-Mezard et al., 2004; Vocanson et al., 2005).

Galectins (Gals) are β-galactoside–binding animal lectins characterized by shared consensus amino acid sequences in the carbohydrate-recognition domain (Liu and Rabinovich, 2010). Gal-1 was the first described Gal family member and is expressed by many tissues (Camby et al., 2006). Hence, Gal-1 is synthesized and secreted by many different types of cells, such as activated T and B cells, regulatory T cells (Tregs), macrophages, dendritic cells, and γδ T cells (Sundblad et al., 2017).

The role of Gal-1 in inflammation has been explored in several in vitro and in vivo experimental models (Auvynet et al., 2013; Corrêa et al., 2017; Iqbal et al., 2011; Rabinovich et al., 2000; Zanon et al., 2015). Exogenous Gal-1 affects the viability, proliferation, and T helper type 1 responses of nonmalignant T cells involved in the progression of cutaneous T-cell lymphoma (Cedeno-Laurent et al., 2012b). Importantly, the administration of exogenous Gal-1 induces IL-10 release by Foxp3$^-$CD4$^+$ T cells, which suppress inflammation (Cedeno-Laurent et al., 2012a, 2010). Moreover, the absence of Gal-1 expression in murine and human CD4$^+$CD25$^+$ T cells reduces their capacity to control the proliferation of effector CD4$^+$ T cells (Garín et al., 2007). However, the differential role of endogenous Gal-1 in the function of regulatory and effector T lymphocyte subsets in CHS has not been addressed.

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Abbreviations: ACD, allergic contact dermatitis; CD, contact dermatitis; CHS, contact hypersensitivity; Gal, galectin; OXZ, oxazolone; Treg, regulatory T cell

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In this study, we show that Gal-1–deficient (Gal-1 $^{/}$) mice displayed increased inflammation and CD8^{+} T-cells infiltration in the skin after treatment with oxazolone (OXZ). Gal-1 expression does not regulate the migration of lymphocytes to the inflamed skin as well as does not affect the regulation of CD8^{+} T-cell proliferation by CD4^{+}Foxp3^{+} T cells in CHS model. However, the depletion of CD4^{+} T cells as well as adoptive transfer experiments demonstrate a major role for Gal-1 in CD8^{+} T cells in CHS. Mechanistically, our data indicate that Gal-1 regulates the central memory CD8^{+} T-cell compartment and the secretion of IFN-γ by effector CD8^{+} T cells.

RESULTS

Gal-1$^{-/-}$ mice showed increased ear swelling and inflammation after CHS

To assess the role of endogenous Gal-1 in the regulation of lymphocyte functions in CHS model, OXZ was administered to wild-type (Gal-1$^{+/+}$) and Gal-1–deficient (Gal-1 $^{/}$) mice. Gal-1 $^{/}$ mice showed increased ear swelling and sustained inflammation compared with Gal-1$^{+/+}$ mice (Figure 1a). Flow cytometry analysis showed that OXZ significantly increased the total numbers of infiltrating CD45^{+} cells and myeloid cells such as neutrophils (CD11b^{+}Ly6G^{+}) and macrophages (CD11b^{+}CD64^{+}) in Gal-1 $^{/}$ mice compared with those in Gal-1$^{+/+}$ mice (Figure 1b). Furthermore, histological analysis revealed that the increase in epidermal and dermal thickness was higher in Gal-1 $^{/}$ mice than in Gal-1$^{+/+}$ mice at 48 hours after the elicitation phase (Figure 1c). These results suggest that the presence of Gal-1 prevents the inflammation induced by OXZ.

We analyzed whether the expression of Gal-1 is differently modulated in lymphoid cells, compared with that in blood and skin tissue, on CHS challenge. The expression of Gal-1 among effector T cells (CD4^{+}, CD8^{+}, γδ^{+}) and Tregs (CD4^{+}Foxp3^{+}) populations was higher in blood than in lymph nodes at 48 hours (Figure 1d). However, the highest expression of Gal-1 was detected in T cells in the inflamed tissue (Figure 1d).

We also evaluated Gal-1 expression in the ear sections of Gal-1$^{+/+}$ mice treated with OXZ at 48 hours after the second challenge by immunofluorescence. The detection of Gal-1–positive cells corresponds mainly to CD45^{+} cells, although the expression of Gal-1 was also observed in vessels but not in keratinocytes (Figure 1e).

Gal-1 deficiency increases skin effector T lymphocytes after elicitation phase of CHS

We analyzed the lymphoid T-cell subsets in Gal-1$^{+/+}$ and Gal-1 $^{/}$ mice expressing IL-17—GFP and Foxp3^{+} RFP in steady state. These experiments demonstrated that Gal-1 $^{/}$ mice are similar to Gal-1$^{+/+}$ mice in terms of the total number and frequency of T-cells populations (CD8^{+}, CD4^{+}, and CD4^{+}Foxp3^{+}) in blood, skin, lymph nodes, and spleen (Supplementary Figure S1a–d). In the thymus, the absence of Gal-1 did not cause any significant difference in the number of CD4^{+}CD8^{+} T cells, single-positive cells, or natural Tregs (CD4^{+}Foxp3^{+} cells) (Supplementary Figure S1e).

To analyze the role of Gal-1 in the sensitization phase, Gal-1$^{+/+}$ and Gal-1 $^{/}$ mice were treated with OXZ in the abdomen (Supplementary Figure S2a). Immune cell populations were analyzed in the lymph nodes after 48 hours and 5 days. We observed that after the first challenge with the hapten, Gal-1 $^{/}$ mice displayed the same frequency and total number of CD4^{+}, CD8^{+}, and Tregs as Gal-1$^{+/+}$ mice (Supplementary Figure S2b and c). Moreover, the neutrophilic infiltration found in the skin after 48 hours of the first challenge with OXZ was similar between both genotypes (Supplementary Figure S2d). Overall, these results indicated that Gal-1 mainly controls the inflammatory response induced after the second challenge with OXZ.

γδ^{+}IL-17^{+} T cells are relevant mediators of skin inflammatory diseases such as ACD (Jiang et al., 2017) and psoriasis (Cibrian et al., 2020, 2016). To address the role of Gal-1 in γδ T cells in the CHS model, we studied this population using the gating strategy indicated in Figure 2a. The number of dermal γδ T cells but not dendritic epidermal γδ T cells increased by day 7 after the elicitation phase in Gal-1 $^{/}$ mice in comparison with that in Gal-1$^{+/+}$ mice (Figure 2b). Interestingly, kinetic analyses of lymphocyte infiltration of OXZ-treated ears demonstrated that Gal-1 deficiency increased the total number of effector CD8^{+} (Figure 2b) and γδ^{+}IL-17^{+} (Figure 2c) T cells at day 7 after treatment. In contrast, Gal-1 $^{/}$ mice were not significantly different from Gal-1$^{+/+}$ in terms of the numbers of CD4^{+} and CD4^{+}Foxp3^{+} cells over time (Figure 2d). These data indicate that Gal-1 $^{/}$ mice develop a sustained inflammation at day 7 after OXZ treatment compared with Gal-1$^{+/+}$ mice, likely owing to an increase of CD8^{+} and IL-17–secreting γδ T cells.

Gal-1$^{-/-}$ and Gal-1$^{+/+}$ T cells display a similar migratory ability to inflamed skin

Gal-1 can be found in circulation as a soluble protein as well as can be expressed by endothelial cells (Norling et al., 2009; Thijssen et al., 2008). To study whether the Gal-1–mediated effect in CHS is related to the adhesion and migration of T cells to OXZ-treated skin, we induced CHS in the presence of a blocking anti–ICAM-1 antibody (Kish et al., 2011). We found that Gal-1 $^{/}$ mice still displayed increased inflammation compared with Gal-1$^{+/+}$ mice, ruling out the involvement of ICAM-1 in the anti-inflammatory effect of Gal-1 in the CHS response induced by OXZ (Figure 3a).

To further assess the role of soluble and endothelial Gal-1 in the migration of immune cells to inflamed skin and lymph nodes, wild-type cells from draining lymph nodes of OXZ-treated mice were intravenously injected in Rag1 $^{/}$ Gal-1 $^{/}$ mice or Rag1 $^{/}$ Gal-1$^{+/+}$ mice (Figure 3b). Flow cytometry data revealed that Gal-1$^{+/+}$ T cells (CD4^{+} and CD8^{+}) similarly migrate to the ears of Rag1 $^{/}$ Gal-1 $^{/}$ – and Rag1 $^{/}$ Gal-1$^{+/+}$–recipient mice (Figure 3c). Furthermore, circulating or endothelial Gal-1 expression does not play a relevant role in CD4^{+} and CD8^{+} T-cells entrance or exit to the lymph nodes (Figure 3d).

Endogenous expression of Gal-1 in T-cell compartment regulates the development of CHS

To ascertain whether the deletion of Gal-1 in bone marrow–derived lymphoid and myeloid cells could recapitulate the enhanced and sustained inflammation observed in the CHS model, we analyzed the inflammation in CD45.1 Gal-1$^{+/+}$ mice lethally irradiated and bone marrow transferred with Gal-1$^{+/+}$ or Gal-1 $^{/}$ cells (Figure 4a). We found that the absence of Gal-1 in hematopoietic cells recapitulates the

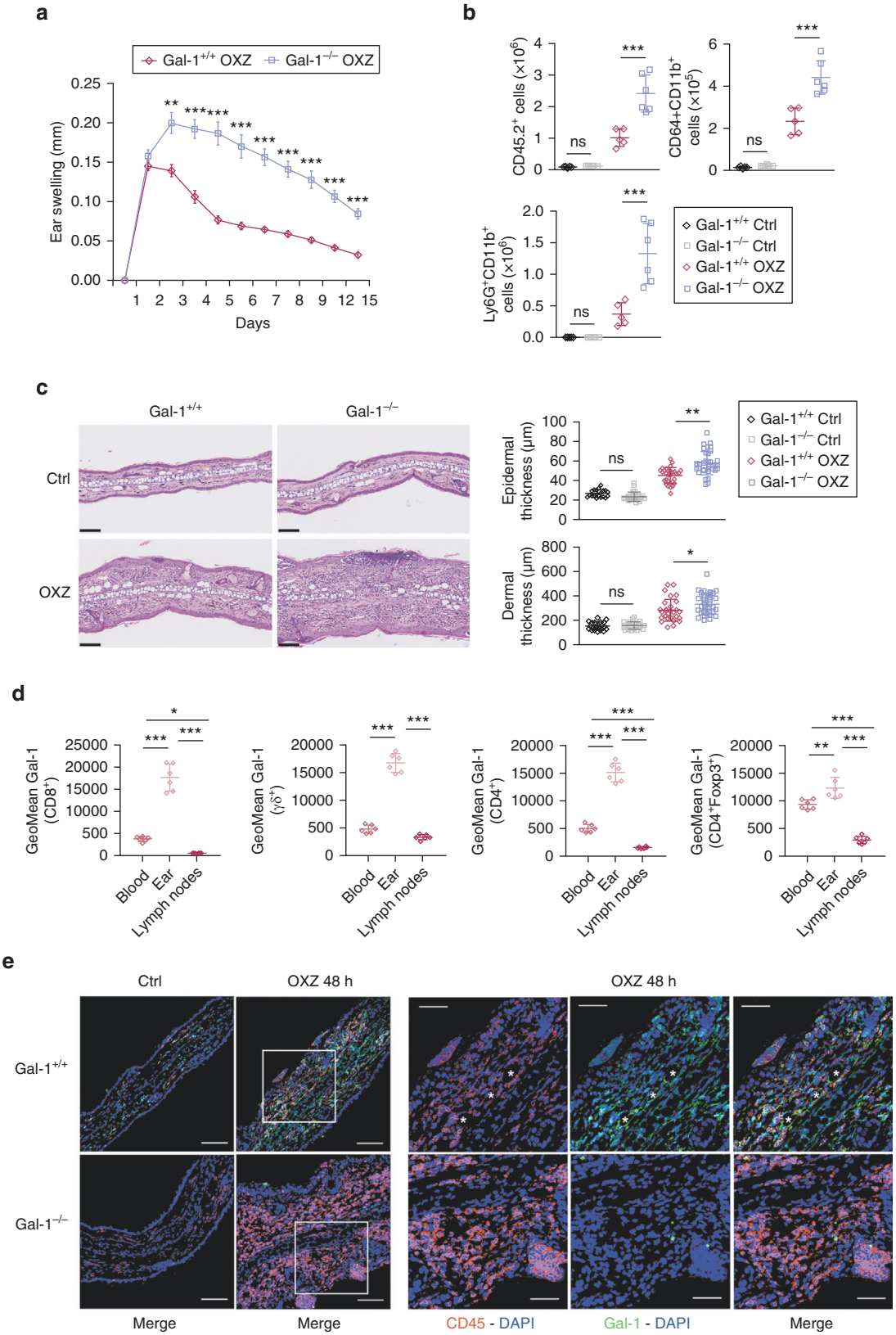

Figure 1. Gal-1$^{-/-}$ mice display increased ear swelling and inflammation after CHS challenge. (**a**) Ear swelling in Gal-1 $^{/}$ and Gal-1$^{+/+}$ mice after OXZ. (**b**) CD45.2^{+} cells, neutrophils (Ly6G^{+}CD11b^{+}), and macrophages (CD64^{+}CD11b^{+}) abundance. (**c**) H&E sections of OXZ-treated ears of Gal-1$^{+/+}$ and Gal-1 $^{/}$ mice (48 h) (left). Epidermal and/or dermal thickness values (right). (**d**) Gal-1 expression in CD8^{+}, γδ^{+}, CD4^{+}, and CD4^{+}Foxp3^{+} T cells after 48 h of OXZ are shown. (**e**) Immunofluorescence of Gal-1 (green) and CD45 (red) in the ear sections. Nuclei were stained with DAPI (blue). Vessels (white asterisk) and zoom areas (right) (white box) are indicated. Bars = 100 µm and 50 µm. Data (mean SD) from one experiment of three are shown. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; one-way ANOVA was used for **b**, **c**, and **d** or two-way ANOVA for **a** with the Bonferroni post hoc test. CHS, contact hypersensitivity; Ctrl, control; Gal-1, galectin-1; h, hour; ns, not significant; OXZ, oxazolone.

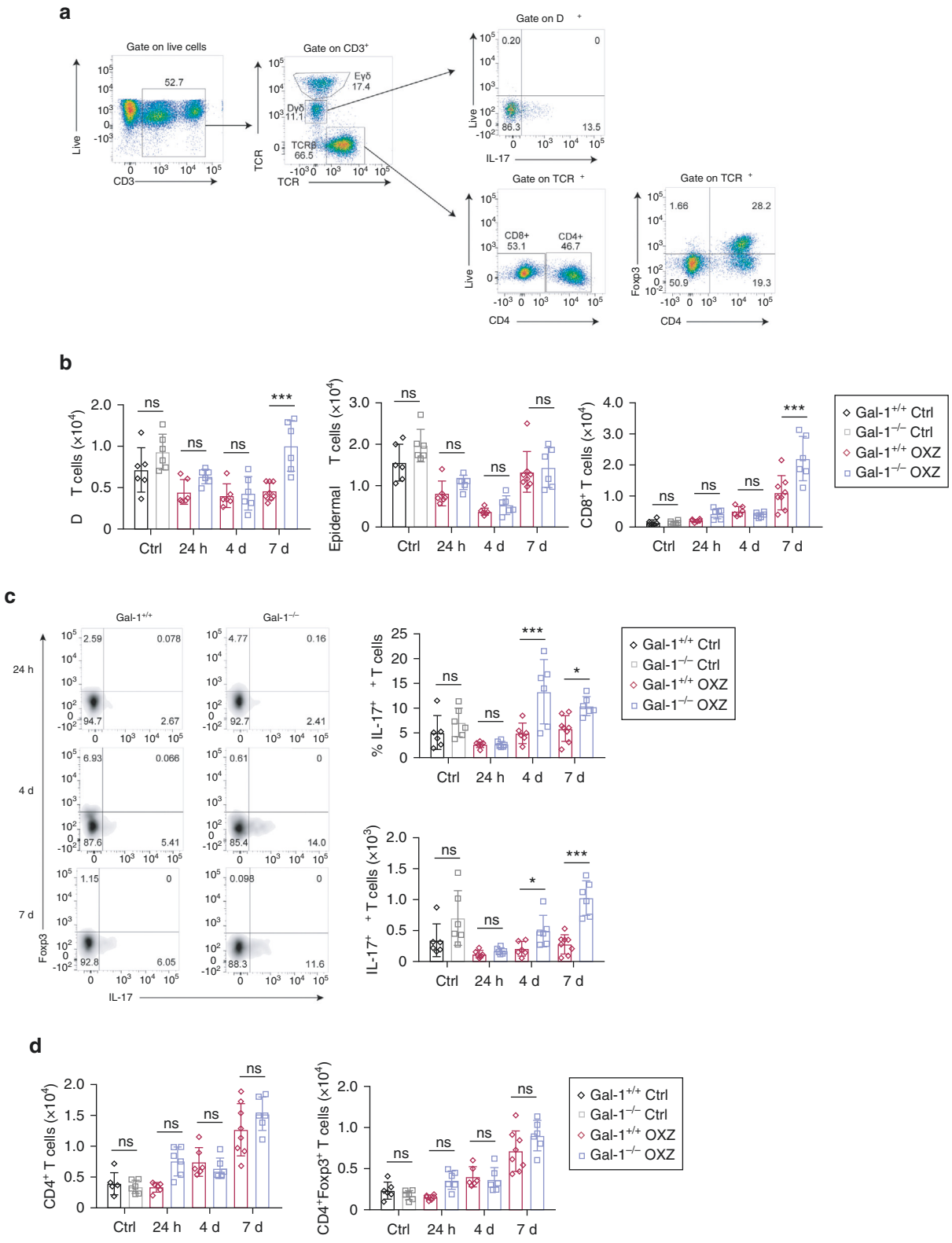

Figure 2. Increased effector T-cells recruitment in the absence of Gal-1 expression. (**a**) Gating strategy applied to skin $CD3^+$ live-gated cells. (**b**) The total cell numbers of $D\gamma\delta^+$, $E\gamma\delta^+$, and $CD8^+$ T cells detected in the skin of OXZ-treated or -untreated (Ctrl) Gal-1 $^{/}$ and Gal-$1^{+/+}$ mice are shown in the graphs. (**c**) Density plots (left), frequencies, and total numbers of IL-$17^+\gamma\delta^+$ T cells (right) quantified in the ears. (**d**) The total cell numbers of $CD4^+$ T cells and $CD4^+Foxp3^+$ T cells in the skin of Gal-1 $^{/}$ and Gal-$1^{+/+}$ mice are shown. Individual data (mean SD) from one representative experiment of three are shown. *$P < 0.05$, ***$P < 0.001$; two-way ANOVA with Bonferroni post hoc test. Ctrl, control; d, day; Dγδ, dermal γδ; Eγδ, epidermal γδ; Gal-1, galectin-1; h, hour; ns, not significant; OXZ, oxazolone.

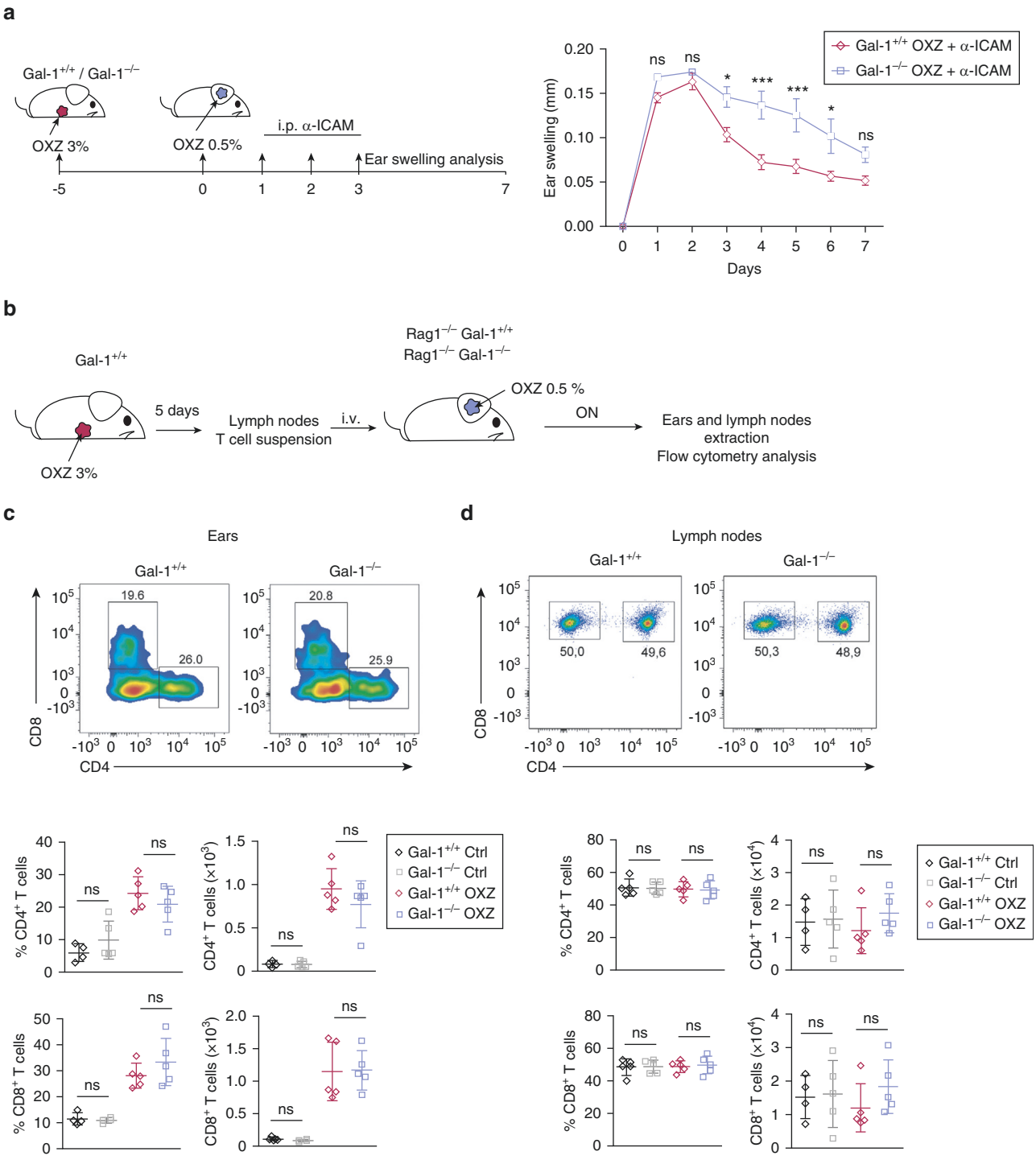

Figure 3. Gal-1 expression does not determine differential migration of T cells to inflamed skin. (**a**) Ear thickness of Gal-1$^{+/+}$ and Gal-1 $^{/}$ mice treated with OXZ and anti–ICAM-1 antibody. (**b**) Rag1 $^{/}$ Gal-1 $^{/}$ and Rag1 $^{/}$ Gal-1$^{+/+}$ mice were sensitized by applying 0.5% OXZ on the right ear (both sides) and were injected with Gal-1$^{+/+}$ T-cell suspension isolated from OXZ-treated mice. Representative plots of CD4^{+} and CD8^{+} T-cells populations of (**c**) ears and (**d**) lymph nodes from Rag1 $^{/}$ Gal-1 $^{/}$ – and Rag1 $^{/}$ Gal-1$^{+/+}$–recipient mice. Individual values of frequencies (left) and total cell numbers (right) are shown in the graphs. Individual data (mean SD) from one representative experiment of three are shown. *$P < 0.05$, ***$P < 0.001$; one-way ANOVA was used for **c** and **d** and two-way ANOVA was used for **a** with Bonferroni post hoc test. Ctrl, control; Gal-1, galectin-1; i.p., intraperitoneal; i.v., intravenous; ns, not significant; ON, overnight; OXZ, oxazolone.

sustained inflammation observed in Gal-1 full–deficient mice (Figure 4b).

To directly assess whether endogenous Gal-1 expression in T cells mediates CHS, we transferred CD45.2^{+} T lymphocytes of OXZ-treated Gal-1 $^{/}$ and -Gal-1$^{+/+}$ mice into CD45.1^{+}Gal-1$^{+/+}$–recipient mice (Figure 4c). After the OXZ challenge, mice receiving Gal-1 $^{/}$ T cells displayed more sustained inflammation than those transferred with Gal-1$^{+/+}$ T cells, indicating that endogenous expression of Gal-1 in T cells but not in the myeloid compartment is sufficient to control CHS (Figure 4d).

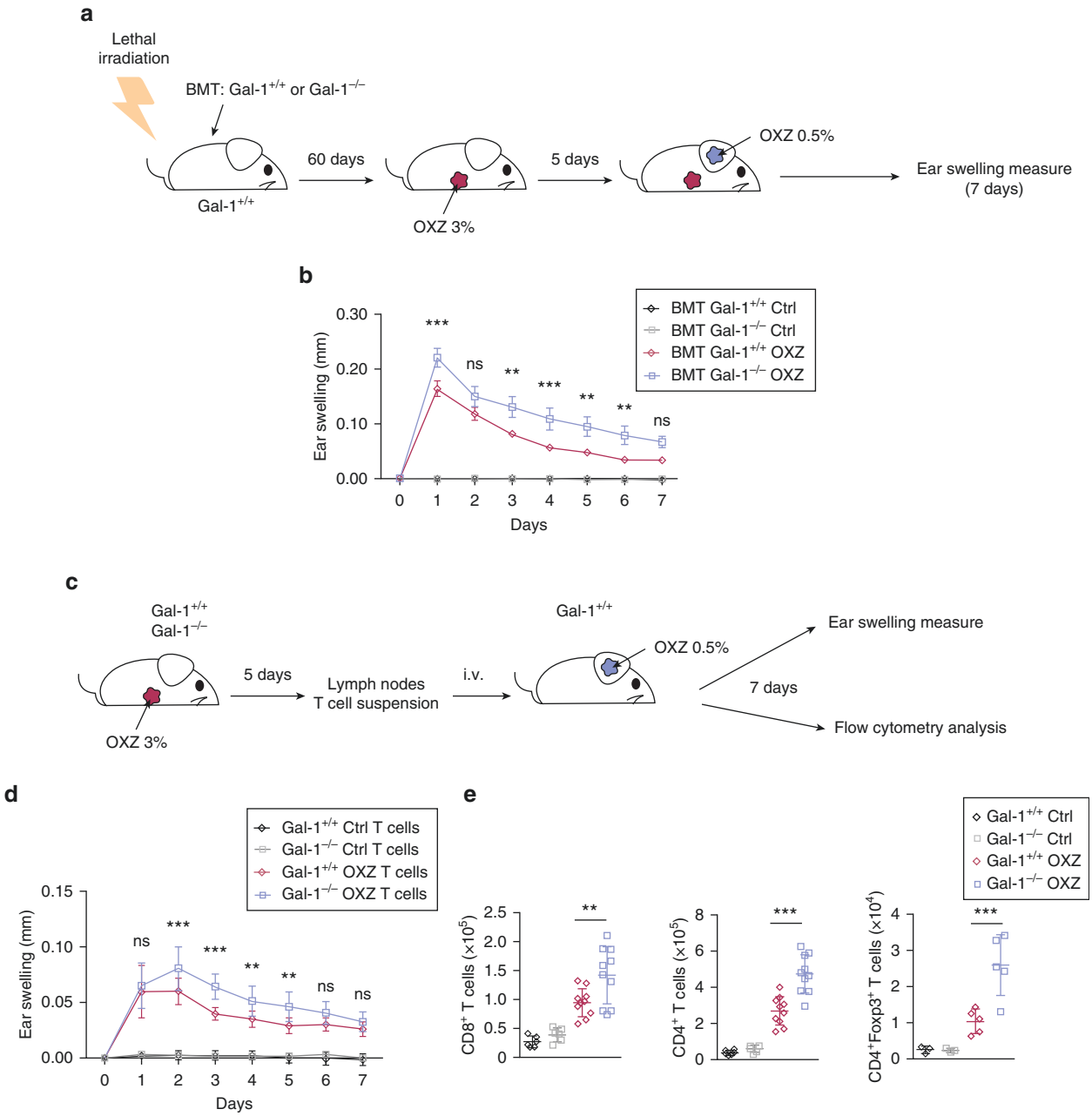

Figure 4. Gal-1 expression plays a major role in T-lymphoid compartment in CHS. (**a**) Wild-type–recipient CD45.1 mice were lethally irradiated and BMT with Gal-1$^{+/+}$ and Gal-1 $^{/}$ cells. CHS response was assessed in chimeric mice after 60 days. (**b**) Increase of ear thickness at different time points. (**c**) Wild-type–recipient mice injected with T cells from OXZ-challenged Gal-1 $^{/}$ and Gal-1$^{+/+}$ mice were treated with OXZ. (**d**) Ear thickness increase is represented. (**e**) The total numbers of CD8^{+}, CD4^{+}, and CD4^{+}Foxp3^{+} T cells in lymph nodes are shown. Individual data (mean SD) from one experiment of three are shown. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; one-way ANOVA was used for **e** and two-way ANOVA was used for **b** and **d** with Bonferroni post hoc test. BMT, bone marrow transplanted; CHS, contact hypersensitivity; Ctrl, control; Gal-1, galectin-1; i.v., intravenous; ns, not significant; OXZ, oxazolone.

Furthermore, flow cytometry analysis demonstrated higher numbers of CD45.2^{+}Gal-1 $^{/}$ CD4^{+}, CD8^{+}, and Tregs in lymph nodes (Figure 4e). These results confirm that the absence of endogenous Gal-1 increases the reactivation of T lymphocytes after the second challenge with OXZ, which promotes the development of the disease.

CD4^{+}Foxp3^{+} T cells are functional in the absence of Gal-1 expression

To address whether the expression of Gal-1 in CD4^{+}Foxp3^{+} Tregs accounts for the phenotype observed in Gal-1 $^{/}$ mice, adoptive transfer of CD4^{+}Foxp3^{+} or CD4^{+}Foxp3 T lymphocytes isolated from OXZ-treated Gal-1 $^{/}$ and -Gal-1$^{+/+}$ mice into Gal-1$^{+/+}$–recipient mice were conducted (Figure 5a). After the OXZ challenge, mice receiving CD4^{+}Foxp3^{+} T cells from both genotypes showed a similarly reduced inflammatory response, suggesting that the regulatory capacity of CD4^{+} Foxp3^{+} cells is not affected by the deletion of Gal-1 in CHS model. Besides, mice receiving effector CD4^{+}Foxp3^{-}Gal-1 $^{/}$ and Gal-1$^{+/+}$ T cells displayed a similarly increased inflammatory response (Figure 5b).

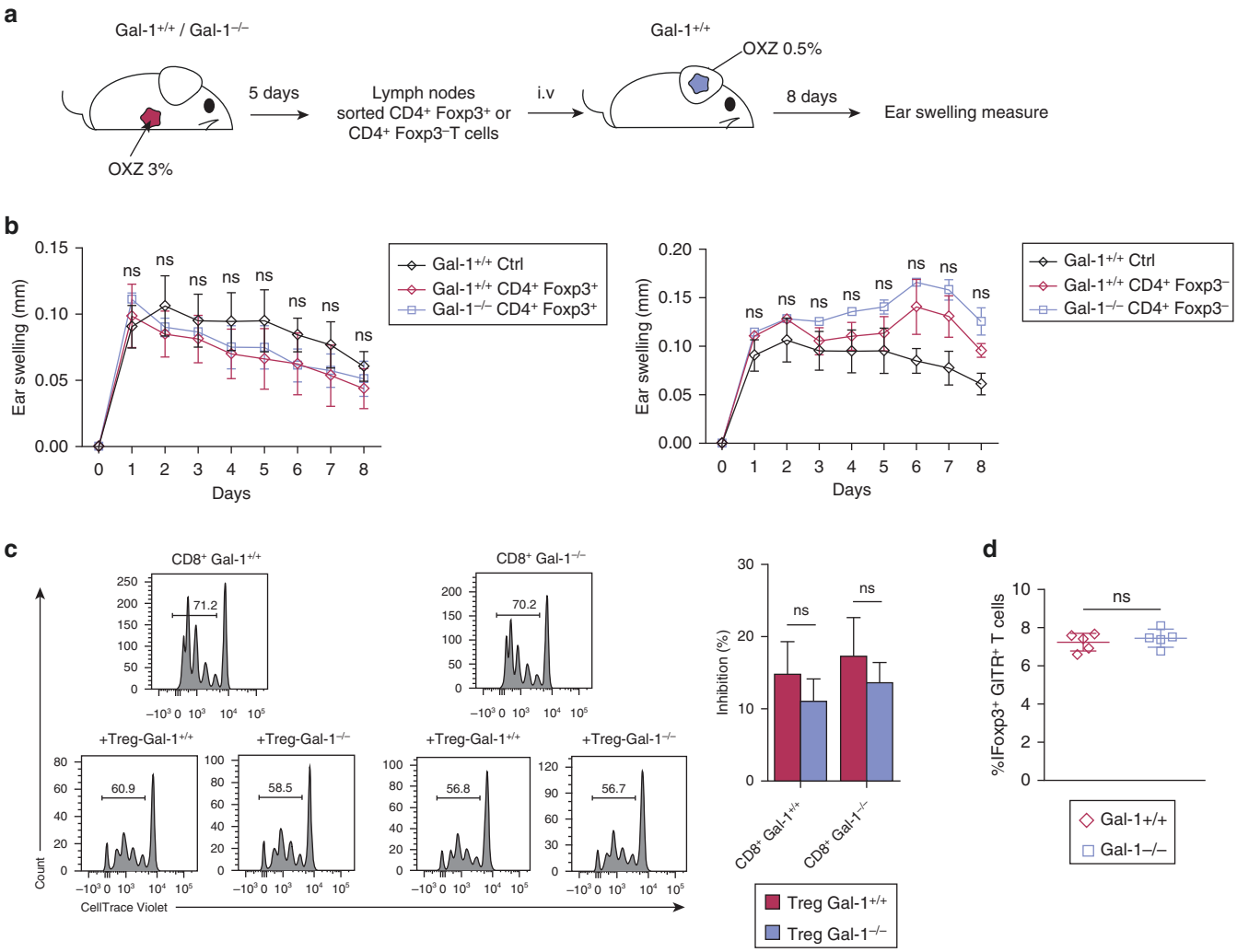

Figure 5. CD4$^+$ Foxp3$^+$ T cells are functional in the absence of Gal-1 expression. (**a**) Wild-type–recipient mice transferred with sorted CD4$^+$Foxp3$^+$ or CD4+Foxp3$^-$ T cells from OXZ-challenged Gal-1 $^{/}$ and Gal-1$^{+/+}$ mice were treated with OXZ. (**b**) Ear thickness increase is shown. (**c**) Histograms (upper) of Gal-1$^{+/+}$ or Gal-1 $^{/}$ CD8$^+$ T-cell proliferation alone or cocultured with Gal-1 $^{/}$ or Gal-1$^{+/+}$ Treg are shown. The percentage of inhibition is shown in the graph (bottom). (**d**) The frequency of Foxp3$^+$GITR$^+$CD4$^+$ T lymphocytes in Gal-1$^{+/+}$ or Gal-1 $^{/}$ mice after sensitization is represented in the graph. Individual data (mean SD) from one representative experiment of three are shown. One-way ANOVA was used for **c** and **d** and two-way ANOVA was used for **b** with the Bonferroni post hoc test. Ctrl, control; Gal-1, galectin-1; GITR, glucocorticoid-induced TNF receptor; i.v., intravenous; ns, not significant; OXZ, oxazolone; Treg, regulatory T cell.

We evaluated the ability of CD4$^+$Foxp3$^+$ T cells derived from OXZ-treated Gal-1 $^{/}$ and Gal-1$^{+/+}$ mice to control CD8$^+$ T-cell proliferation ex vivo. Inhibition of CD8$^+$ T-cell proliferation was similarly mediated by CD4$^+$Foxp3$^+$ T cells from Gal-1 $^{/}$ and Gal-1$^{+/+}$ mice (Figure 5c). In addition, the absence of Gal-1 did not seem to modify the expression of glucocorticoid-induced TNF receptor in CD4$^+$Foxp3$^+$ T cells (Figure 5d). Overall, these results indicate that the deletion of Gal-1 in CD4$^+$ T cells, either effector or regulatory cell, does not affect the CHS response induced by OXZ.

Gal-1–mediated control of CHS response is restricted to CD8$^+$ T-cell compartment

To ascertain the relevance of Gal-1 in CD4$^+$ T cells versus that in CD8$^+$ T lymphocytes, in vivo CD4$^+$ T-cell depletion was performed in Gal-1 $^{/}$ and Gal-1$^{+/+}$ mice (Figure 6a). The differential inflammatory responses detected between both genotypes were maintained in the absence of CD4$^+$ T cells (Figure 6b), indicating a major role for Gal-1 in CD8$^+$ T-cell compartment. Besides, the total number of CD8$^+$ T cells and their proliferation response in skin tissue (Figure 6c) and lymph node (Figure 6d) was similar in Gal-1 $^{/}$ and Gal-1$^{+/+}$ mice regarding the presence or absence of CD4$^+$ T cells.

The transfer of CD8$^+$ T cells derived from OXZ-treated Gal-1 $^{/}$ mice was sufficient to recapitulate the phenotype of full Gal-1 $^{/}$ mice (Figure 6e). Interestingly, an increased fraction of CD44$^+$CD62L$^+$CD8$^+$ T cells but not CD44$^+$CD62L$^+$CD4$^+$ T cells corresponding to the central memory compartment was detected on Gal-1 $^{/}$ mice after the sensitization phase (Figure 6f). Although the expression of T-bet, Granzyme B, EOMES, and PD-1 (Figure 6g) is similar in activated CD8$^+$ T cells from Gal-1 $^{/}$ and Gal-1$^{+/+}$ mice, a significant increase of IFN-γ is detected in CD8$^+$ T cells deficient for Gal-1 (Figure 6h). Moreover, a reduced frequency of IL-10$^+$ and IL-4$^+$CD4$^+$ T cells was detected in Gal-1–deficient mice compared with those in Gal-1$^{+/+}$ mice.

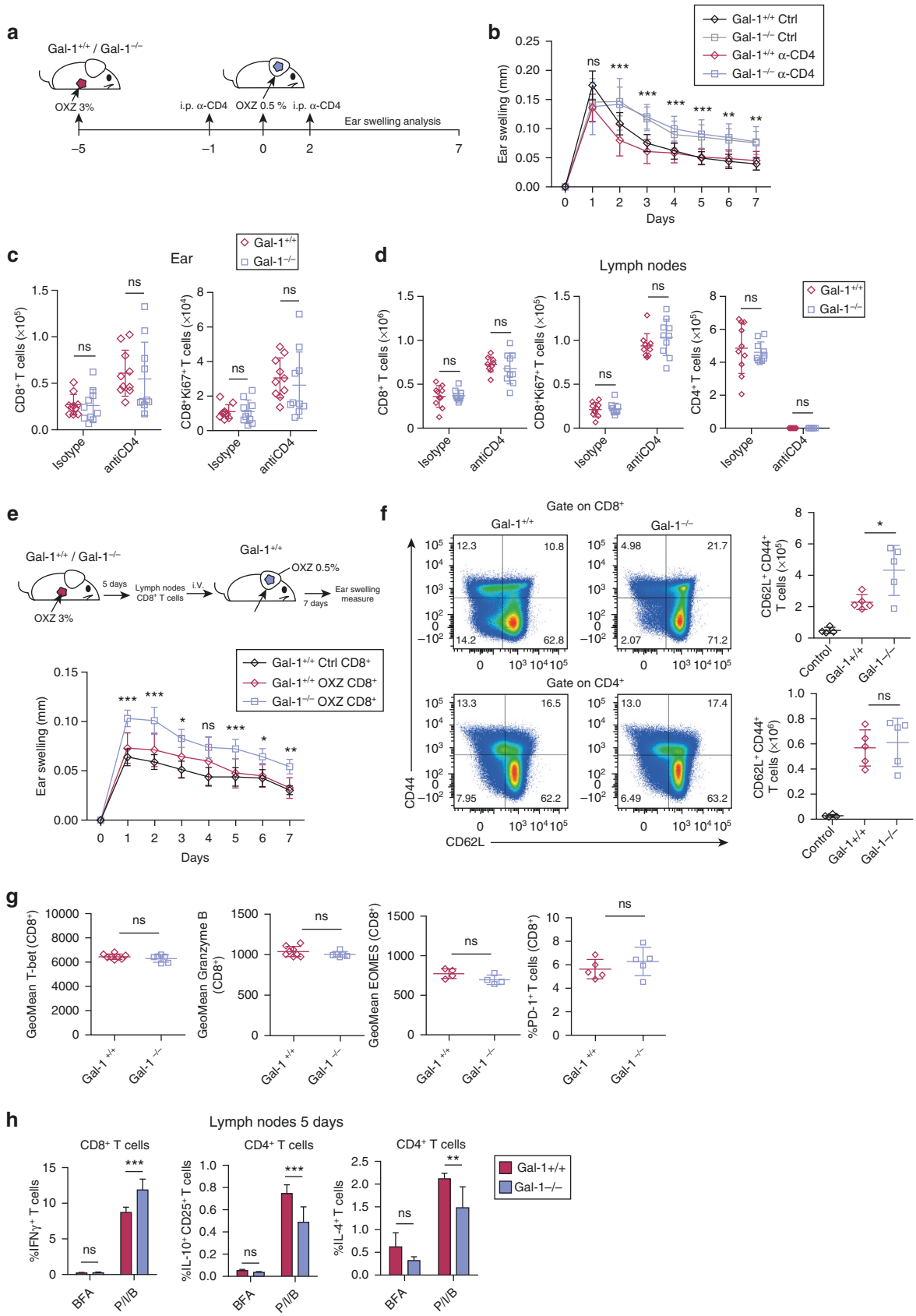

Figure 6. The exacerbated inflammation induced by the absence of Gal-1 is mediated by increased CD8$^+$ T-cell response. (**a**) In vivo anti-CD4 treatment. (**b**) Ear thickness, (**c**) CD8$^+$ and CD8$^+$Ki67$^+$ T cells in the ears, and (**d**) lymph nodes were assessed. (**e**) Mice transferred with CD8+ T cells from OXZ-challenged Gal-1 $^{-/-}$ and Gal-1$^{+/+}$ mice received OXZ (upper). Ear swelling is shown (bottom). (**f**) The representative plots and total number of CD8$^+$CD62L$^+$CD44$^+$ and CD4$^+$CD62L$^+$CD44$^+$ T-cell populations. (**g**) T-bet, granzyme B, EOMES, and PD-1 expression in CD8$^+$ T cells in the lymph nodes. (**h**) The frequency of

These results indicate that Gal-1 deletion increases the development of central memory and IFN-γ–secreting effector $CD8^+$T cells, which induce an exacerbated CHS response.

DISCUSSION

ACD is a common cutaneous pathology induced by an inflammatory reaction in the skin. Frequently, topical and systemic glucocorticosteroids and oral antihistamines are used as a pharmacologic treatment to control the disease (Kaplan et al., 2012). CHS model is a reliable model of ACD and allows to investigate the molecular and cellular mechanisms involved in this pathology. Understanding these mechanisms may lead to developing new treatments for ACD (Kaplan et al., 2012; Martin, 2013).

Pharmacologic interventions with recombinant Gal-1 protein plays an essential immunoregulatory role by inhibiting $CD4^+$ T-cell effector functions in different pathologies such as Crohn's disease (Santucci et al., 2003), multiple sclerosis (Toscano et al., 2007), or asthma (Lv et al., 2019). Previous studies have shown that the interaction between Gal-1 and CD69 modulates T helper type 17 effector cell differentiation and function (de la Fuente et al., 2014). Regarding inflammatory skin disease, the administration of recombinant Gal-1 has been proved to be effective in the control of inflammation (Cedeno-Laurent et al., 2010; Corrêa et al., 2017). However, it is unknown whether endogenous expression of Gal-1 directly influences T-cell subpopulations function and whether Gal-1 from the nonlymphoid compartment contributes to CHS. In this study, we directly demonstrate that the endogenous expression of Gal-1 in effector $CD8^+$ T cells but not in $CD4^+$ T cells plays an important role in the development of CHS model. The development of central memory $CD8^+$ T cells as well as the secretion of IFN-γ by effector $CD8^+$ T cells is increased in the absence of Gal-1. In contrast, the regulatory capacity of Tregs does not appear to be affected in CHS by the genetic deletion of Gal-1.

The relevance of Gal-1 on cellular recruitment and adhesion has been characterized in polymorphonuclear cells in several in vitro and in vivo experimental models (Auvynet et al., 2013; Gil et al., 2010; La et al., 2003). However, whether Gal-1 expression directly affects the migration of activated $CD4^+$ and $CD8^+$ T cells to lymph nodes or inflamed tissue has not been ruled out. Our observations indicate that Gal-1 expressed by endothelial cells does not participate in the modulation of T-cell migration because $CD4^+$ and $CD8^+$ T cells expressing Gal-1 are similarly recruited to the CHS site in Gal-1 $^{-/-}$ and Gal-$1^{+/+}$–recipient mice. The interaction of LFA-1 and ICAM-1 is important to regulate T-cell migration to inflamed tissues (Smith et al., 2007; Verma and Kelleher, 2017). ICAM-1 blockade dampens not only T-cell crawling, adhesion, and transmigration in vitro but also reduces T-cell migration from inflamed ears to the drained lymph nodes in CHS model (Teijeira et al., 2017). However, neutralization of ICAM-1 does not prevent the enhanced inflammation mediated by the absence of Gal-1.

Our data showed that Gal-1 function in T-lymphocyte–dependent inflammatory response after OXZ treatment is confined to the elicitation phase. In the development of CD, not only αβ T cells but also γδ T cells and NK T cells play relevant roles (Askenase, 2001). The role of γδ T cells in CHS is controversial because some studies demonstrate that TCRδ $^{-/-}$ mice develop more inflammation than wild-type mice (Guan et al., 2002), but other authors observed less ear swelling in the absence of γδ T cells (Jiang et al., 2017). IL-17–secreting γδ T cells are increased in Gal-1–deficient mice after the elicitation phase, indicating its potential role in the inflammatory response. However, dermal and epidermal γδ T cells are radioresistant populations in chimeric mice transplanted with bone marrow cells from Gal-1 $^{-/-}$ and Gal-$1^{+/+}$ mice (Jiang et al., 2017), where the phenotype of full deficient mice is recapitulated. These data demonstrate that the expression of Gal-1 on γδ T cells does not exert a key role in the development of CHS model. Moreover, chimeric mice experiments using Gal-$1^{+/+}$ recipient mice also ruled out the potential effect of circulating soluble Gal-1 protein in the mechanism of control of skin inflammation.

Memory $CD8^+$ T cells are antigen-experienced cells that show an improved response to the second challenge in comparison with naive cells. The relationship between central memory $CD8^+$ T cells and effector $CD8^+$ T cells detected in the skin during inflammation has been established in CHS model as well as in viral infection (Gaide et al., 2015; Mbitikon-Kobo et al., 2009; Osborn et al., 2019). Our data demonstrate that the expression of Gal-1 controls the generation of central memory $CD8^+$ T cells and the secretion of IFN-γ, which account for the exacerbated skin inflammation. Hence, this work highlights the protective role of endogenous Gal-1 expression in $CD8^+$ T lymphocytes in the control of CHS-induced skin inflammation.

MATERIALS AND METHODS

Mice

Mice expressing IL-17-GFP and Foxp3-RFP proteins (kindly provided by Richard A. Flavell's Laboratory, Yale University, New Haven, CT) were backcrossed with Gal-$1^{+/+}$ or Gal-1 $^{-/-}$ mice (C57BL/6 background) (Jackson Laboratory, Bar Harbor, ME). Rag1 $^{-/-}$ (C57BL/6 background) (Jackson Laboratory) mice were backcrossed with Gal-$1^{+/+}$ and Gal-1 $^{-/-}$ mice. CD45.1 C57BL/6 mice were purchased (Jackson Laboratory). Male and female, age-matched mice (aged 8–12 weeks) were used. All mice were kept in pathogen-free conditions at the animal facility of Centro Nacional de Investigaciones Cardiovasculares, Madrid, Spain. Experimental procedures were approved by the local Committee for Research Ethics and were in accordance with Spanish and European guidelines.

$CD8^+$IFN-γ^+, $CD4^+$IL-$10^+$$CD25^+$, and $CD4^+$IL-4^+ T cells in the lymph nodes. Data (mean SD) from one experiment out of three are shown. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; two-tailed unpaired Student *t*-test was used for **g**, one-way ANOVA was used for **c**, **d**, **f**, and **h**, and two-way ANOVA was used for **b** and **e** with the Bonferroni post hoc test. BFA, brefeldin A; Ctrl, control; Gal-1, galectin-1; i.p., intraperitoneal; i.v., intravenous; ns, not significant; OXZ, oxazolone; P/I/B, phorbol 12-myristate 13-acetate, ionomycin, and brefeldin A mix.

CHS model

The shaved abdomen of mice was treated with 200 µl of OXZ (Sigma-Aldrich, St. Louis, MO) solution (3%) dissolved in ethanol on day 1. On day 5, a second challenge was induced using 20 µl OXZ (0.5%) on both sides of the ear. As a control, mice were painted with 20 µl vehicle alone (ethanol). Ear swelling, measured as an increase of ear thickness, was assessed daily.

To carry out in vivo ICAM-1 blockade experiments, mice were treated with 150 µg of an anti-CD54 antibody or the isotype control (Bio X Cell, Lebanon, NH) 3 days after elicitation challenge.

$CD4^+$ T cells were depleted in vivo using rat anti-mouse CD4 antibody (clone GK1.5; rIgG2b; Bio X cell) (1 mg per mouse, intraperitoneal) injected at 24 hours before and 48 hours after the second challenge. Control mice were treated with rIgG2b isotype control (Bio X cell).

Adoptive transfer experiments

Gal-$1^{+/+}$ mice treated with OXZ (3%) were killed on day 5, and T-cell suspension was obtained from lymph nodes. Rag1 $^{/}$ Gal-1 $^{/}$ or Rag1 $^{/}$ Gal-$1^{+/+}$ mice treated with OXZ in the ears were injected (intravenously) with the T-cell suspension (one donor to one recipient). Ears and lymph nodes were analyzed after 24 hours. Similarly, Gal-$1^{+/+}$ and Gal-1 $^{/}$ mice were treated with OXZ, and T-cell suspension from lymph nodes was obtained and injected intravenously in $CD45.1^+$Gal-$1^{+/+}$ mice. Control mice were treated with vehicle.

$CD4^+$ $Foxp3^+$ (2.5 10^6 cells per mouse) and $CD4^+$ $Foxp3^-$ (50 x 10^6 cells per mouse) T cells and $CD8^+$ cells (40 10^6 cells per mouse) were purified (Stemcell Technologies, Vancouver, Canada) and sorted for Treg separation (FACSAria Cell Sorter, BD Biosciences, San Jose, CA). Purified cells from OXZ-treated Gal-$1^{+/+}$ and Gal-1 $^{/}$ mice were injected (intravenously) to $CD45.1^+$ Gal-$1^{+/+}$ mice treated with OXZ in the ears. Control mice were injected with an equal number of T cells from nonsensitized mice.

Chimeric mice

CD45.1 mice (Gal-$1^{+/+}$) were lethally irradiated and transplanted with 5 10^6 Gal-$1^{+/+}$ or Gal-1 $^{/}$ bone marrow cells. After reconstitution (60 days), mice were challenged with OXZ as described earlier.

Flow cytometry

Tissues were dissected and grated through a nylon mesh (70 µm; BD Biosciences) to obtain single-cell suspensions. Ears were digested with the following mix: Liberase TM Research grade (Sigma-Aldrich) (0.08 mg/ml), collagenase IV (0.5 mg/ml), and DNAse (Sigma-Aldrich) (100 µg/ml) in RPMI medium supplemented with fetal bovine serum (1%) for 35 minutes at 37 C. The tissue was mechanically disrupted using 7 mm of stainless-steel beads (Life Technologies, Grand Island, NY) in a TissueLyser LT (Qiagen, Hilden, Germany) one 3-minute cycle (20 oscillations/s). Cell suspensions were incubated with anti-FcRII/III (clone 2.4G2) before staining with specific antibodies (Supplementary Table S1). Stimulation of T cells were performed with phorbol 12-myristate 13-acetate (50 ng/mL; Sigma-Aldrich), ionomycin (500 ng/mL; Sigma-Aldrich) in the presence of brefeldin A (GolgiStop, 1 µg/mL; BD Biosciences), by at least 4 hours. Intracellular stainings were conducted with the Fixation/Permeabilization Solution Kit (BD Biosciences). Dead cell staining was done with Fixable Yellow Viability Dye (Molecular Probes, Eugene, OR). Absolute count of cells was conducted using BD Trucount Tubes (BD Biosciences). Cell samples were acquired in a FACSCanto and LSRFortessa Flow Cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star, Woodburn, OR).

Skin histology and immunofluorescence staining

Samples were fixed in formaldehyde and embedded in paraffin. Slices were stained with H&E and digitalized. Epidermis and dermis thickness were measured every 100 µm using NDP Viewer software (Hamamatsu, Japan).

Deparaffinized sections were boiled in Tris-EDTA buffer: 10 mM Tris base, 1 mM EDTA solution, 0.05% Tween 20, pH 9.0. After blocking (PBS-5%-chicken serum), sections were incubated with primary antibodies (Supplementary Table S1) followed by secondary antibodies: chicken anti-rabbit AlexaFluor488 and chicken anti-goat AlexaFluor647. Nuclei were counterstained with DAPI. Images were captured in a Zeiss LSM 700 Confocal microscope and analyzed with LSM image browser software (Zeiss, Jena, Germany).

In vitro assay of Treg function

Mouse cells were cultured in RPMI medium supplemented with fetal bovine serum (5%), 25 mmol/l of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, antibiotics, sodium pyruvate, and β-mercaptoethanol. Single-cell suspensions of lymph nodes from OXZ-treated Gal-$1^{+/+}$ and Gal-1 $^{/}$ mice were obtained. Tregs were isolated and purified as $CD25^+$ T cells using EasySep Release Mouse Biotin Positive Selection Kit (Stemcell Technologies). From the negative fraction ($CD25^-$ T cells), the $CD8^+$ T cells were purified (Stemcell Technologies) and labeled using CellTrace Violet Cell Proliferation Kit (Thermo Fisher Scientific, Waltman, MA). Cells were seeded (10 Treg:1 CD8) in plate coated with anti-CD3 (2 µg/ml) and anti-CD28 (0.5 µg/ml) (Tonbo Biosciences, San Diego, CA). $CD8^+$ T-cells proliferation was assessed at 60 hours.

Statistical analysis

Statistical significance was assessed by two-tailed unpaired Student *t*-test, one-way ANOVA, or two-way ANOVA with Bonferroni multiple comparisons post hoc test, as required. All analyses were performed with GraphPad software (San Diego, CA).

Data availability statement

No datasets were generated during this study.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization: FSM, MF; Formal Analysis: RCG, DC, NFG, MRH, MLS; Funding Acquisition: FSM; Investigation: RCG, DC, NFG, MRH, MLS; Methodology: RCG, DC; Resources: HDLF, MNN, MF; Visualization: RCG, DC; Writing - Original Draft Preparation: RCG, DC, FSM; Writing - Review and Editing: RCG, DC, FSM, HDLF, MNN, MF

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2020.10.020.

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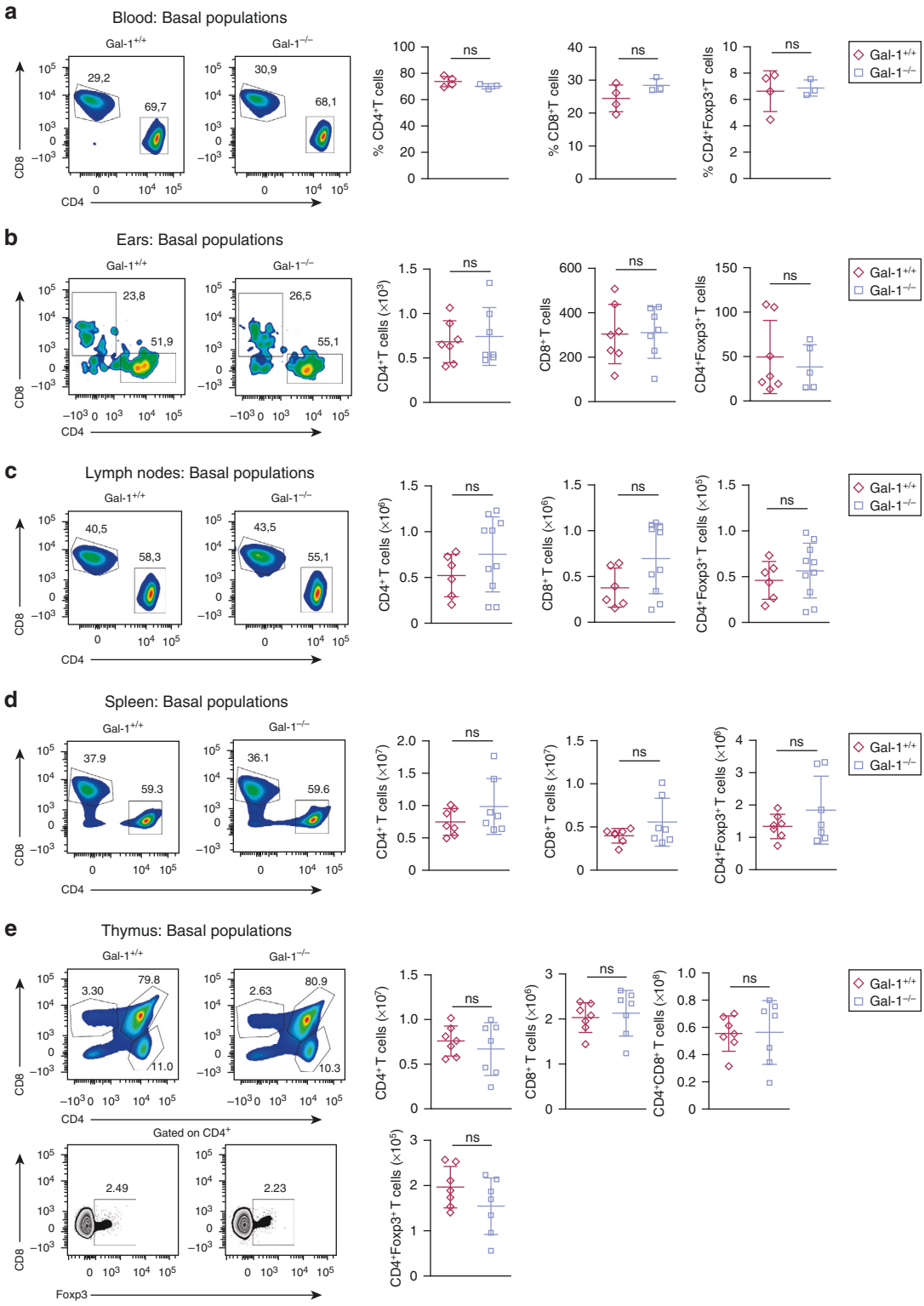
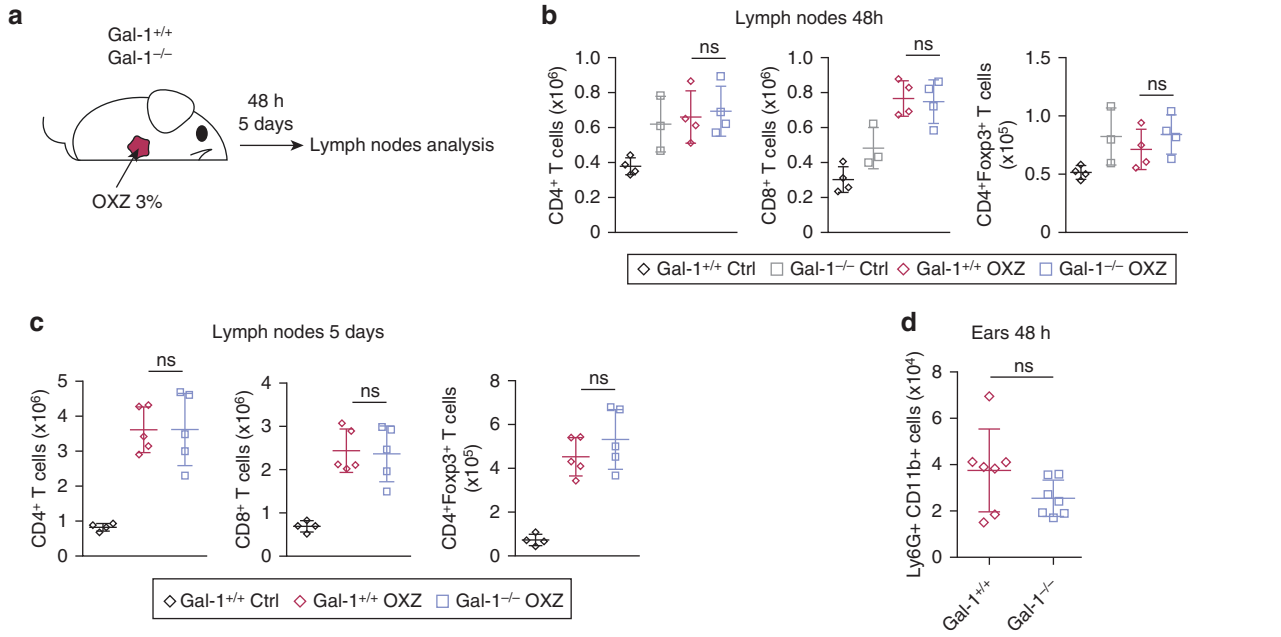


Supplementary Figure S1. Gal-1 genetic deletion does not alter the populations of CD4$^+$ and CD8$^+$ T cells in homeostasis. (**a**) Density plots (left) and frequency (right) of CD4$^+$, CD8$^+$, and CD4$^+$Foxp3$^+$ cells from TCRαβ$^+$ gated cells in the blood. The density plots (left) and total numbers of CD4$^+$, CD8$^+$, and CD4$^+$Foxp3$^+$ (right) from TCRαβ$^+$ cells in the (**b**) ears, (**c**) lymph nodes, and (**d**) spleen in homeostasis in Gal-1 $^{/}$ and Gal-1$^{+/+}$ mice. (**e**) The density plots (left) and total cell numbers of the populations described earlier and the CD4$^+$CD8$^+$ cells (right) from CD45$^+$ cells in the thymus are shown in the graphs. Data from one representative experiment of three are shown (mean SD). Unpaired *t*-test was used for analysis. Gal-1, galectin-1; ns, not significant.



Supplementary Figure S2. Gal-1 does not regulate the sensitization phase of CHS. (**a**) Gal-1 $^{/}$ and Gal-1$^{+/+}$ mice were treated with OXZ in the abdomen and killed after 48 h and 5 days. Flow cytometry analysis of the total number of CD4^{+}, CD8^{+}, and CD4^{+}Foxp3^{+} T cells in lymph nodes at (**b**) 48 h and (**c**) 5 days after OXZ treatment. (**d**) The total numbers of neutrophils (CD11b^{+}Ly6G^{+}) detected in the skin at 48 h after the sensitization phase are shown. Individual data (mean SD) from one representative experiment of three are shown. One-way ANOVA with Bonferroni post hoc test was used for b–c; unpaired *t*-test was used for the analysis in **d**. CHS, contact hypersensitivity; Ctrl, control; Gal-1, galectin-1; h, hour; ns, not significant; OXZ, oxazolone.

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Supplementary Table S1. List of Used Antibodies

Specificity	Reactivity	Clone	Fluorochrome	Origin	Dilution	Catalog No
CD3e	Mouse	145-2C11	APC/BV421/purified	BD Biosciences, San Jose, CA/Tonbo Biosciences, San Diego, CA	1:200/1:250	553066/561416/70-0031
CD4	Mouse	RM4-5	APC/BV421/biotin	Tonbo Biosciences, San Diego, CA/BD Biociences, San Jose, CA	1:200	20-0042/740007/553045
CD8	Mouse	53-6.7	APC Fire750/Pe-Cy7	BioLegend, San Diego, CA/Tonbo Biosciences, San Diego, CA	1:200	100766/60-0081
CD25	Mouse	PC61.5	Biotin	Tonbo Biosciences, San Diego, CA	1:200	30-0251
CD28	Mouse	37.51	Purified	Tonbo Biosciences, San Diego, CA	1/1,000	70-0281
CD45	Mouse	Polyclonal Goat IgG	Purified	Research and Diagnostic Systems, Minneapolis, MN	1:40	AF114-SP
CD45.2	Mouse	104	Pe-Cy7	eBiocience, San Diego, CA	1:200	560696
CD11b	Mouse	M1/70	FITC/biotin	BD Biociences, San Jose, CA	1:200	553310/553309
CD11c	Mouse	HL3	PE/biotin	BD Biociences, San Jose, CA	1:200	557401/553800
CD64	Mouse	X54-5/7.1	APC	BioLegend, San Diego, CA	1:200	139311
EOMES	Mouse	Dan11mag	PE	eBiocience, San Diego, CA	1:200	12-4875-80
FcRII/III (CD16/CD32)	-	2.4G2	Purified	Tonbo Biosciences, San Diego, CA	1:100	70-0161
Gal-1	Mouse	D608T/Polyclonal Rabbit IgG	Biotinylated/purified, IHC formulated	Research and Diagnostic Systems, Minneapolis, MN/Cell Signaling, Danvers, MA	1:100	BAF1245/13888
GITR	Mouse	DTA-1	Pe-Cy7	BioLegend, San Diego, CA	1:200	126317
Granzyme B	Mouse	NGZB	Pe-Cy7	eBiocience, San Diego, CA	1:200	25-8898-80
IFN-γ	Mouse	XMG1.2	FITC/APC	eBiocience, San Diego, CA	1:200	11-7311-82/17-7311-82
IL-4	Mouse	11B11	PE	BioLegend, San Diego, CA	1:200	504104
IL-10	Mouse	JES5-16E3	BV421	BioLegend, San Diego, CA	1:200	505022
Ly6G	Mouse	1A8	PE	BD Biociences, San Jose, CA	1:200	551461
PD-1	Mouse	29F.1A12	BV421	BioLegend, San Diego, CA	1:200	135217
γδTCR	Mouse	GL3	PerCP/Cy5.5/biotin	eBiocience, San Diego, CA	1:200	560696/13-5711-82
TCRαβ	Mouse	H57-597	APC-Cy7	BioLegend, San Diego, CA	1:200	109220
T-bet	Mouse	4B10	PerCP/Cy5.5	eBiocience, San Diego, CA	1:200	45-5825-82

Abbreviations: APC, allophycocyanin; BV, brilliant violet; Cy, cyanine dye; FITC, fluorescein isothiocyanate; Gal-1, galectin-1; IHC, immunohistochemically; No, number; PE, phycoerythrin; PerCP, peridinin chlorophyll protein.

Targeting L-type amino acid transporter 1 in innate and adaptive T cells efficiently controls skin inflammation

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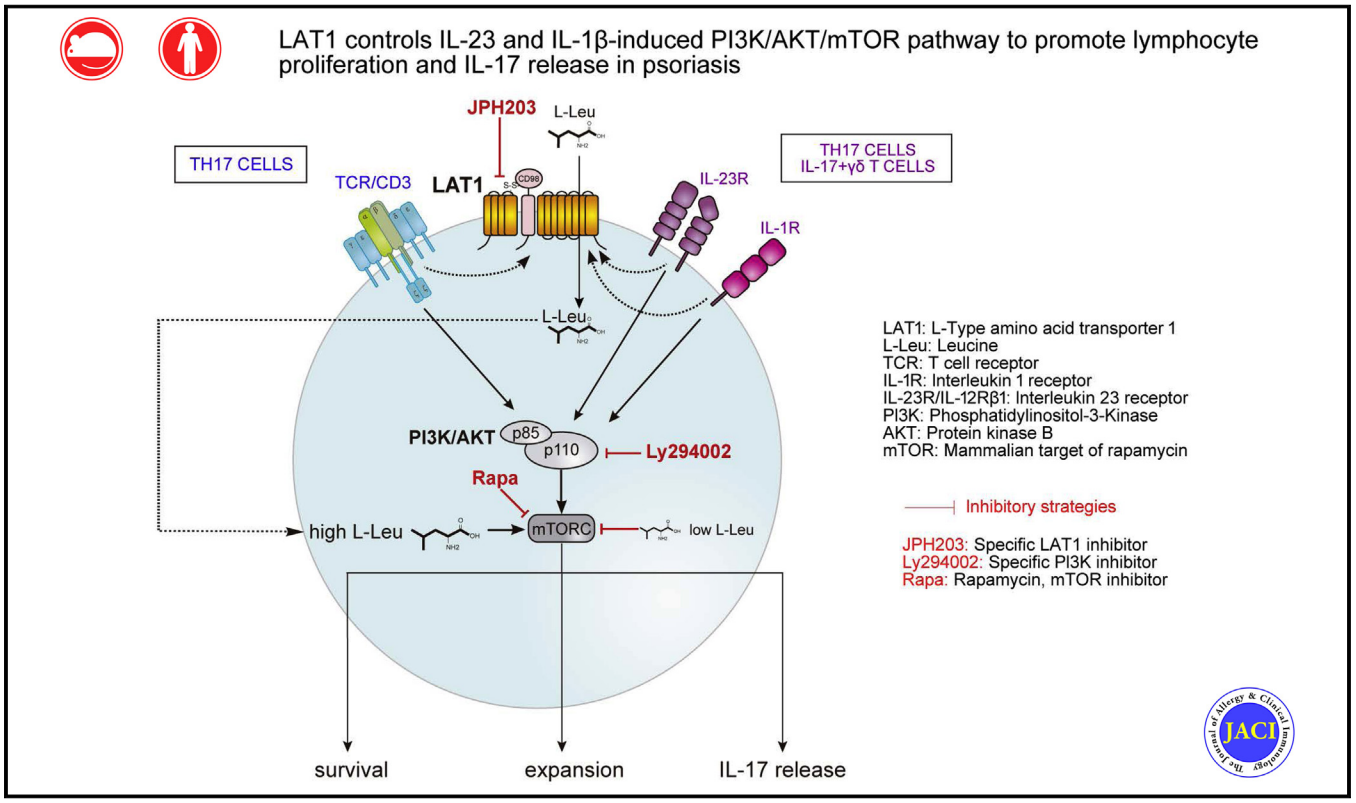
GRAPHICAL ABSTRACT



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Background: Psoriasis is a frequent inflammatory skin disease that is mainly mediated by IL-23, IL-1β, and IL-17 cytokines. Although psoriasis is a hyperproliferative skin disorder, the possible role of amino acid transporters has remained unexplored.

Objective: We sought to investigate the role of the essential amino acid transporter L-type amino acid transporter (LAT) 1 (SLC7A5) in psoriasis.

Methods: LAT1 floxed mice were crossed to Cre-expressing mouse strains under the control of keratin 5, CD4, and retinoic acid receptor–related orphan receptor γ. We produced models of skin inflammation induced by imiquimod (IMQ) and IL-23 and tested the effect of inhibiting LAT1 (JPH203) and mammalian target of rapamycin (mTOR [rapamycin]).

Results: LAT1 expression is increased in keratinocytes and skin-infiltrating lymphocytes of psoriatic lesions in human subjects and mice. LAT1 deletion in keratinocytes does not dampen the inflammatory response or their proliferation, which could be maintained by increased expression of the alternative amino acid transporters LAT2 and LAT3. Specific deletion of LAT1 in γδ and CD4 T cells controls the inflammatory response induced by IMQ. LAT1 deletion or inhibition blocks expansion of IL-17–secreting $\gamma4^+\delta4^+$ and CD4 T cells and dampens the release of IL-1β, IL-17, and IL-22 in the IMQ-induced model. Moreover, inhibition of LAT1 blocks expansion of human γδ T cells and IL-17 secretion by human CD4 T cells. IL-23 and IL-1β stimulation upregulates LAT1 expression and induces mTOR activation in IL-17$^+$ γδ and T_H17 cells. Deletion or inhibition of LAT1 efficiently controls IL-23– and IL-1β–induced phosphatidylinositol 3-kinase/AKT/mTOR activation independent of T-cell receptor signaling.

Conclusion: Targeting LAT1-mediated amino acid uptake is a potentially useful immunosuppressive strategy to control skin inflammation mediated by the IL-23/IL-1β/IL-17 axis. (J Allergy Clin Immunol 2020;145:199-214.)

Key words: *L-type amino acid transporter 1, SLC7A5, psoriasis, γδ T cells, T_H17, mammalian target of rapamycin*

Abbreviations used

AHR:	Aryl hydrocarbon receptor
BrdU:	Bromodeoxyuridine
DMSO:	Dimethyl sulfoxide
FC:	Flow cytometry
H&E:	Hematoxylin and eosin
IL-1R:	IL-1 receptor
IMQ:	Imiquimod
K5:	Keratin 5
LAT:	L-type amino acid transporter
L-Leu:	L-leucine
mTOR:	Mammalian target of rapamycin
NF-κB:	Nuclear factor κ-light-chain-enhancer of activated B cells
PI3K:	Phosphatidylinositol 3-kinase
PMA:	Phorbol 12-myristate 13-acetate
P-S6:	Phospho-S6 ribosomal protein
RORγt:	Retinoic acid receptor–related orphan receptor γ
TCR:	T-cell receptor
WT:	Wild-type

Psoriasis is a common skin disorder characterized by formation of focal plaques of inflamed and raised skin with squamous appearance.[1] The affected portions of the skin show massive keratinocyte proliferation and prominent infiltration of immune cells, suggesting that psoriasis is a primary disorder of the skin immune system.[1-3] As in patients with other chronic inflammatory diseases, the proinflammatory cytokines IL-23, IL-1β, and IL-17 exert important functions in patients with psoriasis.[4-6]

Human psoriatic skin showed increased numbers of IL-17–releasing γδ and T_H17 cells,[7] as were also seen in murine models induced by topical application of the Toll-like receptor 7/8 agonist imiquimod (IMQ) or IL-23 injections.[8-10] Increased numbers of dermal macrophages, dendritic cells, and keratinocytes secreting IL-1β and IL-23 are also described.[8] IL-23 stimulates the survival and proliferation of T_H17 cells, whereas IL-1 receptor (IL-1R)–deficient mice do not properly display T_H17 cells.[11] However, the molecular mechanism by which IL-23 receptor and IL-1R contribute to the development, survival, and expansion of IL-17 cells remains unclear. Importantly, IL-1β potentiates the effects of IL-23 by inducing expression of IL-23 receptor in both T_H17 and γδ T cells.[11,12]

L-type amino acid transporter (LAT) 1 (SLC7A5) is a 512 amino acid–long, 12-transmembrane protein that mediates sodium-independent large neutral amino acid transport.[13] LAT1 (light chain) forms a heterodimeric complex with CD98/SLC3A2 (heavy chain) through disulfide bonds.[14] Heterodimerization stabilizes and enhances LAT1 transport to the plasma membrane, making it fully functional.[15] The alternative amino acid transporters LAT2/SLC7A8, LAT3/SLC43A1, and LAT4/SLC43A2 can also mediate large neutral amino acid uptake.[16] However, LAT1 is the main L-leucine (L-Leu) transporter expressed in T-cell receptor (TCR)–activated T cells[17] and natural killer cells.[18]

The expression of LAT1 is increased in many cancer cells,[19] including malignant skin lesions.[20] LAT1 is the main mediator of L-Leu uptake in malignant cells, and its usefulness as a target to inhibit cancer proliferation has been explored. Accordingly, the novel LAT1-specific inhibitor JPH203 has emerged as a promising cancer therapy that blocks the phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway.[21]

Previously, we showed that CD69 regulates L-tryptophan uptake through LAT1, which contributes to aryl hydrocarbon receptor (AHR) activation in skin-resident γδ T cells.[22] In addition, LAT1 regulates L-Leu transport in CD4 T cells, controlling mTOR activation induced by TCR signaling.[17] However, a direct contribution of LAT1 to skin inflammation *in vivo* remains unexplored.

Here we use a conditional (floxed) LAT1 knockout mouse ($LAT1^{fl/fl}$)[17] to assess the role of LAT1 in keratinocyte proliferation and in CD4 and γδ T-cell function in an *in vivo* model of psoriasis induced by IMQ. Our results show that LAT1 deletion does not affect keratinocyte proliferation, whereas it effectively prevents expansion of IL-17–releasing immune cells. The data herein demonstrate that LAT1 inhibition shuts down the PI3K/AKT/mTOR axis induced by IL-23 and IL-1β in immune cells, becoming a promising novel strategy to control the inflammatory response that underlies the onset and course of psoriasis.

METHODS

A complete and detailed description of the methods used in this study are presented in the Methods section in this article's Online Repository at www.jacionline.org.

Mice

LAT1 floxed mice have been previously described.[17] CD4-Cre and retinoic acid receptor–related orphan receptor γ (RORγt)–Cre mice were purchased from the Jackson Laboratory (Bar Harbor, Me). Remaining mouse strains, such as Krt5-CreERT2 mice, were kindly provided by different groups.

Human subjects

Skin punch biopsy specimens (3 mm) and blood samples were obtained from patients with psoriasis and healthy volunteers. The study was approved by the Hospital Universitario de La Princesa ethics committee, and all participants provided written informed consent.

Psoriasis model

IMQ-induced[8,23] and an IL-23–induced[22] murine models of skin inflammation were used, as described previously.

Histologic analysis

Skin samples (from mice and human subjects) were processed for hematoxylin and eosin (H&E) staining and analysis of LAT1, LAT2, LAT3, and IL-17 expression by using immunohistochemical and immunofluorescence techniques.

Flow cytometry analysis and *in vitro* stimulation

Single-cell suspensions from mouse skin, blood, and lymph nodes were stained with a mixture of appropriate anti-mouse antibodies and analyzed by using flow cytometry (FC) technique. Total PBMCs or purified CD4 T cells from healthy donors and patients with psoriasis were also analyzed by using FC after staining with corresponding antibodies (see Table E1 in this article's Online Repository at www.jacionline.org). The sequence of used primers are listed in Table E2.

Human and mouse cell suspensions were *in vitro* stimulated with IL-23, IL-1β, or both (10 ng/mL each) to analyze cytokine release (IL-17, IL-22, and IFN-γ), proliferation (Ki-67 and bromodeoxyuridine [BrdU]), and phospho-S6 ribosomal protein (P-S6) expression.

Amino acid assessments

The serum L-Leu profile of normal (wild-type [WT]) and *Rag1*$^{-/-}$ mice treated or not with IMQ (for 5 days, 50 mg/d) were measured by using liquid chromatography–tandem mass spectrometry.

Uptake of the ^{3}H-radiolabeled amino acids L-phenylalanine and L-Leu (PerkinElmer, Waltham, Mass) was assessed in activated CD4 T cells from LAT1^{WT}, LAT1$^{\Delta R\gamma t}$, and LAT1$^{\Delta CD4}$ mice, according to the protocol described previously.[22]

Statistical analysis

Results were reported as mean ± SEMs. Statistical evaluations were performed with GraphPad Prism 7 software (GraphPad Software, La Jolla, Calif). Normality of data distribution was assessed by using the Kolmogorov-Smirnov test. The 2-tailed Student *t* test or Mann-Whitney test was used for comparison of 2 populations. Multiple comparisons were performed by using 1-way or 2-way ANOVA, followed by *post hoc* (Bonferroni) tests. A *P* value of less than .05 was considered significant.

RESULTS

Expression of the essential amino acid transporter LAT1 in psoriasis

To assess the relevance of essential amino acids in psoriasis, we evaluated levels of L-Leu in normal (WT) and immunodeficient (*Rag1*$^{-/-}$) mice treated or not with IMQ. Circulating levels of L-Leu in *Rag1*$^{-/-}$ mice were increased compared with those in WT mice, suggesting that essential amino acids are likely modulated by adaptive B or T cells in the IMQ model (Fig 1, *A*).

Normal skin is characterized by LAT2 expression, whereas LAT1 mainly appears in several different types of malignant skin lesions.[24,25] Our results clearly showed that LAT1 expression is induced in keratinocytes and lymphocytes in human psoriatic skin (Fig 1, *B* and *C*), as well as in mouse skin after IMQ application (Fig 1, *D*). Further analysis showed that dermal IL-17^{+} cells observed in mice treated with IMQ express LAT1 (Fig 1, *E*).

Considering the high expression of LAT1 in the epidermal layer of patients with psoriasis, we examined the effect of deleting LAT1 in keratinocytes. Mice carrying floxed LAT1 alleles (LAT1$^{fl/fl}$)[17] were crossed with mice expressing the Cre recombinase controlled by the keratinocyte-specific keratin 5 (K5) promoter (K5-CreERT2 mice)[26] and Rosa26-floxed stop tdTomato (Tm) mice.[27] This system fluorescently labels the cells that successfully underwent LAT1 deletion. After tamoxifen administration, mice displaying fluorescently red skin from both genotypes, LAT1^{WT} and LAT1$^{\Delta K5}$, were selected for topical treatment with IMQ. Both groups developed psoriasis after IMQ application independent of LAT1 expression in keratinocytes (Fig 2, *A*). The increase in epidermal thickness was similar in both genotypes (Fig 2, *B*). Neutrophil and macrophage infiltration also increased upon IMQ application independent of LAT1 expression in epidermal cells (Fig 2, *C*). Cre recombinase, as determined by Tm expression, was not detected in inflammatory cells (data not shown).

Analysis of Tm^{+} keratinocytes (CD49f^{+}) confirmed deletion of LAT1 expression after tamoxifen administration (Fig 2, *D*). The proliferation markers Ki-67 and BrdU revealed that deletion of LAT1 does not affect keratinocyte proliferation (Fig 2, *E*). These results indicate that essential amino acid uptake in these cells does not require LAT1 and could be mediated by other amino acid transporters, such as LAT2 or LAT3, which can also shuttle L-Leu.[16] LAT2 is broadly expressed in keratinocytes in normal human skin and increased in psoriatic lesions (see Fig E1, *A*, in this article's Online Repository at www.jacionline.org). Epithelial cells, such as HaCat and Caco-2 cells, express LAT2 and LAT1, whereas Hela and lymphoid cells only express LAT1 (see Fig E1, *B*). Moreover, LAT2 expression in keratinocytes increased in LAT1$^{\Delta K5}$ and LAT1^{WT} mice after IMQ application (see Fig E1, *C*). Finally, high expression of LAT3 was observed in the basal layer of keratinocytes of normal human skin and psoriatic lesions (see Fig E1, *D*). These data indicate that psoriasis increases the expression of several essential amino acid transporters in keratinocytes to support enhanced proliferation.

IL-17^{+} secreting cells detected in the skin of IMQ-treated mice are mainly Vγ4^{+}δ4^{+} and CD4 T cells.[28] Importantly, the proliferative response of Vγ4^{+}δ4^{+} (Fig 2, *F*) and CD4 (Fig 2, *G*) T cells induced in the draining lymph nodes after IMQ application was similar in both mouse groups independent of LAT1 expression in keratinocytes.

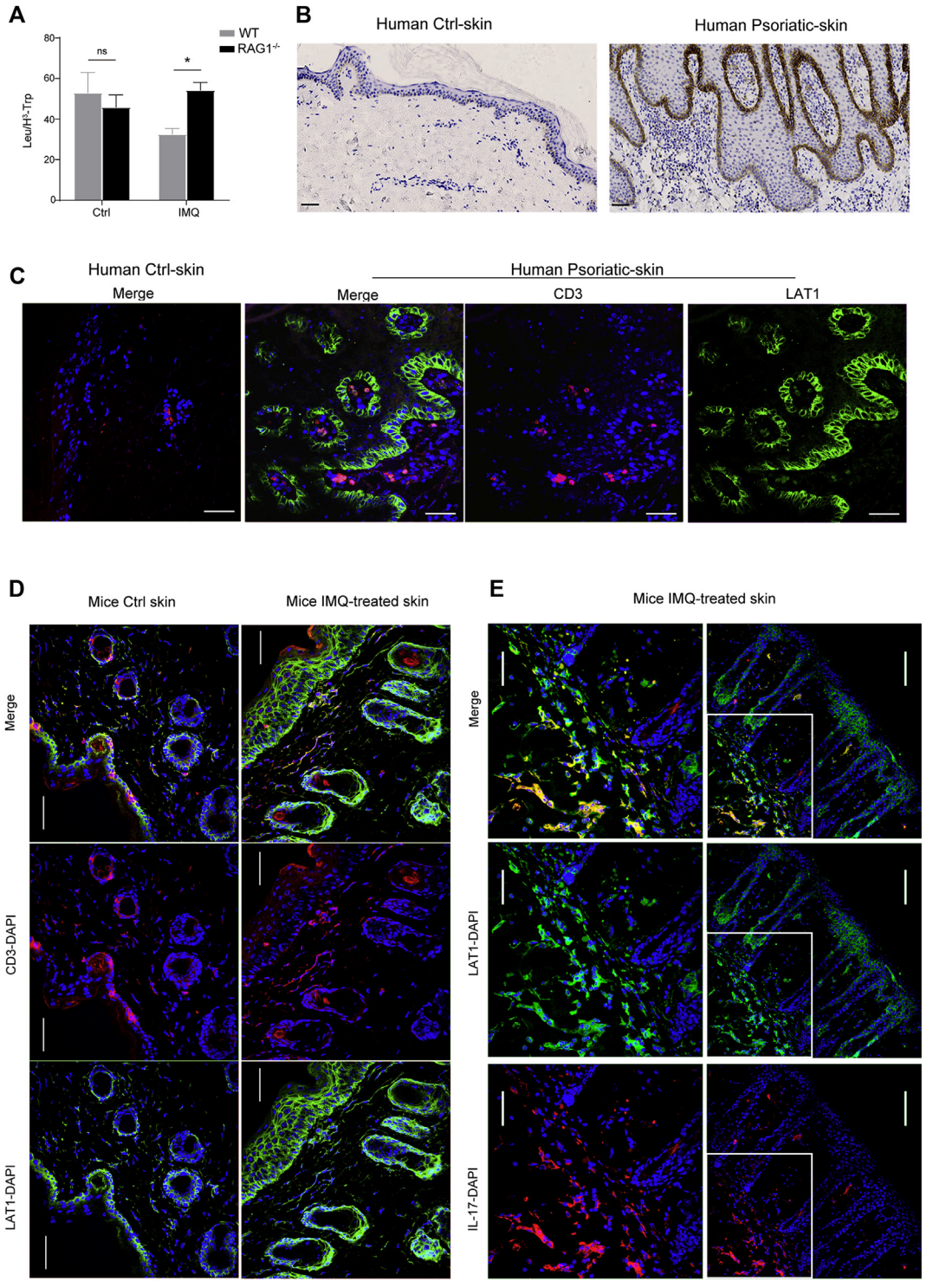

FIG 1. Expression of the amino acid transporter LAT1 is induced in patients with psoriasis and in the IMQ model. **A,** Relative levels of L-Leu detected by using mass spectrometry in sera of healthy *(Ctrl)* and IMQ-treated WT and *Rag1*$^{-/-}$ mice (n = 4-6 mice). *Bars* indicate means ± SEMs. *ns,* Not significant. **P* < .05, 2-tailed unpaired Student *t* test. **B,** LAT1 detection by means of immunohistochemistry in skin biopsy specimens from healthy donors and patients with psoriasis. **C,** Representative immunofluorescence of LAT1 (green) and CD3 (red) in skin samples from healthy donors and patients with psoriasis. **D,** Immunofluorescence of LAT1 (green) and CD3 (red) in dorsal skin of WT mice after IMQ and control skin. **E,** Detection of LAT1 (green) expression in dermal cells secreting IL-17 (red) in mice treated with IMQ for 4 days. Zoom areas *(left)* are indicated by the *white box (right)*. Nuclei were always stained with 4′-6-diamidino-2-phenylindole dihydrochloride (*DAPI*; blue). *Scale bars* = 100 and 50 μm in zoom area. At least 3 skin biopsy specimens of mouse or human origin were simultaneously analyzed in each experiment.

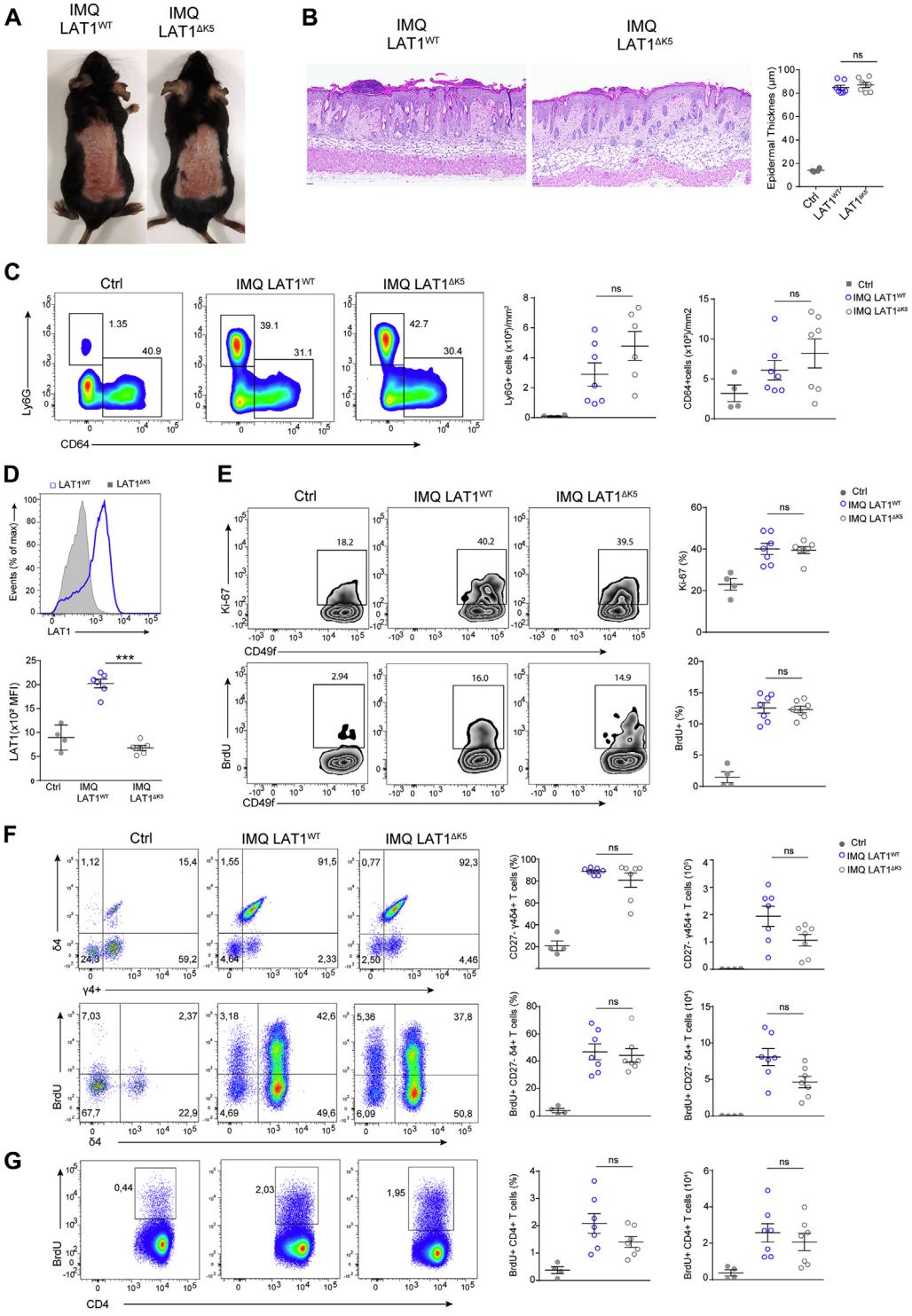

FIG 2. Deletion of LAT1 in keratinocytes does not affect psoriasis induced by IMQ. **A,** Representative pictures of LAT1^{WT} and LAT1$^{\Delta k5}$ mice after IMQ application. **B,** Representative H&E skin sections per group. *Scale bars* = 100 μm. Averaged values of epidermal thickness per mice are shown in the graphic at right. **C,** Representative density plots of skin neutrophil (Ly6G$^+$; *top*) and macrophage (CD64$^+$; *right*) populations on CD45$^+$ live cells. Density values of cells are shown in the graphics. **D,** Histogram *(upper)* and individual values *(bottom)* of LAT1 fluorescence in live keratinocytes. **E,** Density plots *(left)* and individual values of frequencies *(right)* of Ki-67$^+$ *(upper panels)* and BrdU$^+$ *(lower panels)* cells from live keratinocytes. **F,** Dot plots of Vγ4$^+$δ4$^+$ *(upper)* and BrdU$^+$δ4$^+$ *(bottom)* cells from live CD27$^-$ γδ T cells quantified in the lymph nodes. **G,** Dot plots of the frequency of BrdU$^+$ CD4 T cells in lymph nodes. Individual values of frequencies *(left)* and total cell numbers *(right)* are shown in the graphs (Fig 2, *F* and *G*). A representative experiment of 2 is shown (n = 4-7 per group). Data are represented as means ± SEMs. *ns,* Not significant. ***$P < .001$, 1-way ANOVA with the Bonferroni *post hoc* test (Fig 2, *B-G*).

LAT1 deletion in innate and adaptive T cells prevents psoriasis

To simultaneously study the function of LAT1 in adaptive and innate lymphocytes, we crossed LAT1$^{fl/fl}$ mice[17] with RORγt-Cre$^{+/-}$ mice[29] and Rosa26-floxed stop tdTomato mice.[27] Characterization of LAT1$^{\Delta R\gamma t}$ mice revealed that specific deletion of LAT1 in the skin occurs in cells that had expressed and/or were expressing RORγt, including dermal $\gamma\delta$ T cells, skin-resident type 3 innate lymphoid cells, and skin CD4 T cells (see Fig E2, *A*, in this article's Online Repository at www.jacionline.org). Expression of Cre-recombinase was detected in CD27$^-$ $\gamma\delta$ T cells within lymph nodes (see Fig E2, *B*). These are mainly IL-17–secreting cells that also express RORγt.[30] Moreover, because RORγt is also expressed during T-cell development at the immature double-positive stage in the thymus,[31] deletion of LAT1 can occur also in most TCR$\alpha\beta$ T-cell subsets (see Fig E2, *B*). Indeed, *in vitro*–activated CD4 T cells from LAT1$^{\Delta R\gamma t}$ mice did not express LAT1 (see Fig E2, *C*). Also, these cells were smaller and less complex (see Fig E2, D) and displayed defective amino acid uptake (see Fig E2, E).

To ascertain whether deletion of LAT1 in RORγt$^+$ cells could affect skin lymphocyte populations at steady state, we analyzed the distribution of different skin CD3$^+$ T-cell subsets. No significant differences in the frequency or absolute numbers of dermal CD4 and $\gamma\delta$ T cells, which were mostly Tm$^+$, were detected in LAT1$^{\Delta R\gamma t}$ and LAT1^{WT} mice at steady state (see Fig E2, *F*). Epidermal $\gamma\delta$ T cells (dendritic epidermal T cells), which were identified by greater TCR expression, did not express RORγt or Tm and were otherwise unaffected by LAT1 deletion in the other subsets (see Fig E2, *F*).

Compared with LAT1^{WT} mice, induction of psoriasis in LAT1$^{\Delta R\gamma t}$ mice was clearly reduced, with a smaller area affected and an almost complete absence of redness and squamous appearance (Fig 3, *A*). Protection in LAT1$^{\Delta R\gamma t}$ mice was confirmed by analyzing keratinocyte proliferation by means of H&E and Ki-67 staining of skin sections, which was markedly reduced compared with that in LAT1^{WT} mice (Fig 3, *B*). IMQ-treated LAT1$^{\Delta R\gamma t}$ mice also displayed decreased infiltration of neutrophils and monocyte-derived inflammatory macrophages compared with IMQ-treated LAT1^{WT} mice (Fig 3, *C*). Importantly, lower numbers of dermal CD4 and $\gamma\delta$ T-cell populations were observed in the dorsal skin of IMQ-treated LAT1$^{\Delta R\gamma t}$ mice compared with LAT1^{WT} mice (Fig 3, *D*).

Analysis of draining lymphoid cells from IMQ-treated LAT1^{WT} mice showed that IL-17–producing CD27$^-$ $\gamma\delta$ T cells express LAT1 in contrast to CD27$^+$ $\gamma\delta$ T cells, which do not secrete IL-17 (see Fig E3, *A*, in this article's Online Repository at www.jacionline.org). LAT1$^{\Delta R\gamma t}$ mice showed a reduced frequency of CD27$^-$ Tm$^+$ $\gamma\delta$ T cells and lower Ki-67 expression than IMQ-treated LAT1^{WT} mice (Fig 3, *E*). Moreover, application of IMQ increased expression of LAT1 in dermal Vγ4$^+$ and Vγ4$^-$ $\gamma\delta$ T cells in LAT1^{WT} mice (Fig 3, *F*). Similarly, LAT1 expression was mainly increased in CD27$^-$ Vγ4$^+\delta$4$^+$ T cells detected in draining lymph nodes after IMQ application (Fig 3, *G*). These results indicate that deletion of LAT1 affected innate and adaptive lymphocyte expansion induced by IMQ, suggesting its potential role as a therapeutic target in skin inflammation. In addition, LAT1$^{\Delta R\gamma t}$ mice showed less ear thickness, S100A8/9 expression, and neutrophil and macrophage skin infiltration than LAT1^{WT} mice (see Fig E3, *B-D*) in an alternative model of psoriasis induced by intradermal injection of IL-23.

Specific target deletion of LAT1 in CD4 T cells attenuates psoriasis

Although $\gamma\delta$ T cells are essential to develop psoriasis in the IMQ- and IL-23–induced models,[8,10] this population is not the most abundant in patients with psoriasis, in which most of the IL-17–secreting cells are TCR$\alpha\beta$ cells.[32] To determine whether specific deletion of LAT1 in CD4 T cells controls psoriasis development induced by IMQ, we crossed LAT1$^{fl/fl}$ mice with CD4-Cre$^{+/-}$ mice.[33] LAT1 deletion in CD4 T cells was confirmed by using Western blotting and FC analysis (see Fig E4, *A*, in this article's Online Repository at www.jacionline.org). *In vitro*–activated CD4 T cells from LAT1$^{\Delta CD4}$ mice showed reduced amino acid uptake compared with LAT1^{WT} CD4 T cells (see Fig E4, *B*). Importantly, the specific inhibitor of LAT1, JPH203, completely blocked amino acid uptake in CD4 T cells, indicating the relevance of this specific essential amino acid transporter in CD4 T cells.

On treatment with IMQ, LAT1$^{\Delta CD4}$ mice displayed a smaller affected area and reduced redness and squamous appearance compared with LAT1^{WT} mice (Fig 4, *A*). Histologic assessment revealed reduced epidermal thickness in LAT1$^{\Delta CD4}$ mice (Fig 4, *B*). Numbers of infiltrating neutrophils and CD4 T cells, but not Ly6C$^+$ macrophages and $\gamma\delta$ T cells, were attenuated in the skin of LAT1$^{\Delta CD4}$ mice (Fig 4, *C* and *D*).

Expression of LAT1 was increased in CD4 T cells after IMQ application (Fig 4, *E*). Moreover, CD4 T-cell proliferation was induced by IMQ treatment in the lymph nodes of LAT1^{WT} but not of LAT1$^{\Delta CD4}$ mice (Fig 4, *F*). As a control, we tested the proliferation of lymph node CD27$^-$ $\gamma\delta$ T cells, which remained unaffected when LAT1 was deleted in CD4 T cells (see Fig E4, *C*). Transcriptional expression analysis of purified CD4 (Fig 4, *G*) and $\gamma\delta$ (see Fig E4, *D*) T cells from the draining lymph nodes of mice treated with IMQ indicated that deletion of LAT1 in CD4 T cells specifically controls IL-17 mRNA levels in CD4 T cells but not in $\gamma\delta$ T cells.

Transcriptional levels of IL-22 and IFN-γ in CD4 and $\gamma\delta$ T cells after IMQ application were comparable between both genotypes (Fig 4, *G*, and see Fig E4, *D*). IL-10 expression was reduced in CD4 T cells from LAT1$^{\Delta CD4}$ mice compared with LAT1^{WT} mice (Fig 4, *G*) but was similar in $\gamma\delta$ T cells (see Fig E4, *D*). Expression of LAT1 mRNA levels was also increased by IMQ in CD4 T cells from LAT1^{WT} mice (Fig 4, *G*).

Further stimulation with IL-23 and IL-1β of CD4 T cells purified from lymph nodes of IMQ-treated mice showed that LAT1$^{\Delta CD4}$ mice displayed lower levels of IL-17 and IL-22 expression (Fig 4, *H* and *I*). These data indicate that LAT1 controls expansion of CD4 T cells, as well as their ability to secrete IL-17 and IL-22 in response to IL-23 and IL-1β stimulation. Moreover, naive CD4 T cells from LAT1$^{\Delta CD4}$ and LAT1^{WT} mice were differentiated *in vitro* toward the T$_H$17 program by means of addition of IL-6 and TGF-β in combination with IL-23 plus IL-1β. CD4 T cells from LAT1$^{\Delta CD4}$ mice displayed lower numbers of IL-17$^+$ cells and secreted less IL-17 and IL-22 than cells expressing LAT1 (see Fig E4, *E*). Human CD4 T cells from healthy donors were also *in vitro* differentiated toward T$_H$17 with the same cytokine cocktail

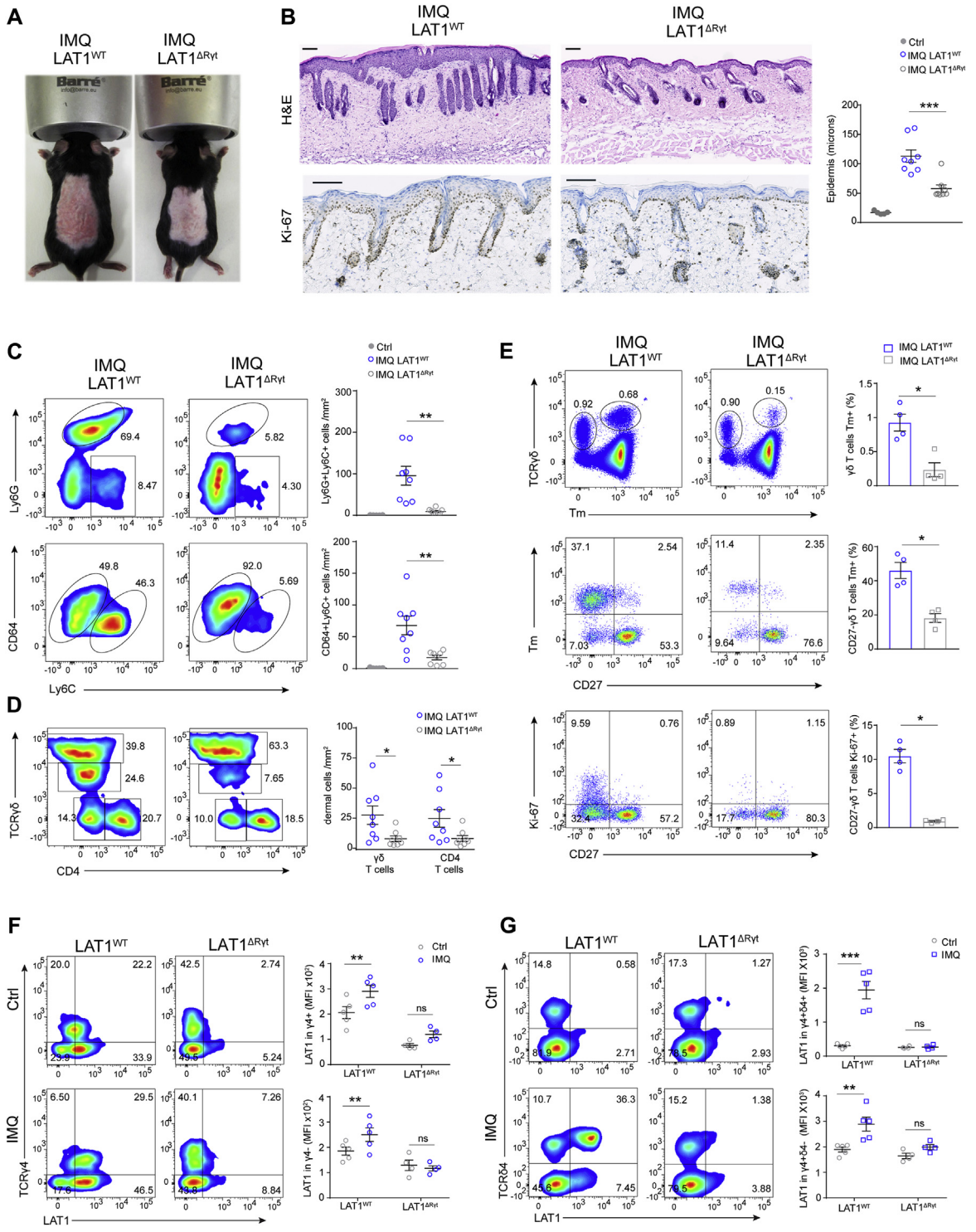

FIG 3. Deletion of LAT1 in innate and adaptive T cells prevents IMQ-induced psoriasis. **A,** Representative pictures of LAT1^{WT} and LAT1$^{\Delta R\gamma t}$ mice after IMQ. **B,** H&E-stained *(top)* and Ki-67–stained *(bottom)* sections. *Scale bars* = 100 μm. Averaged values of epidermal thickness are shown *(right)*. **C,** Skin neutrophils (Ly6G$^+$L6C$^+$; *upper*) and resident (CD64$^+$Ly6C$^-$) versus inflammatory macrophages (CD64$^+$Ly6C$^+$; *bottom*) were quantified. **D,** Dermal CD4 (*bottom* and *right*) and γδ T cells (*middle* and *left*) and epidermal γδ T cells (*upper* and *left*) were analyzed. Absolute numbers of cells are shown (Fig 3, *C* and *D*, *right*). **E,** Dot plots of Tm$^+$ γδ T cells from CD3$^+$ cells *(upper)*, Tm$^+$ CD27$^-$ cells *(middle)*, and CD27$^-$ γδ Ki-67$^+$ T cells *(bottom)* from total γδ T cells are shown. Frequency values are indicated *(right)*. **F** and **G,** LAT1 expression in Vγ4$^+$ *(top)* and Vγ4$^-$ *(bottom)* dermal T cells (Fig 3, *F*) and lymph node Vδ4$^+$ *(top)* and Vδ4$^-$ *(bottom)* T cells (Fig 3, *G*) in normal and IMQ-treated mice. Data are represented as means ± SEMs. Results of 2 independent experiments are represented as absolute numbers per square millimeter (Fig 3, *C*; n = 4). Individual data from one representative experiment of 2 were shown (Fig 3, *D-F*; n = 4-5). *ns*, Not significant. $^*P < .05$, $^{**}P < .01$, and $^{***}P < .001$, 1-way ANOVA (Fig 3, *C*) or 2-way ANOVA (Fig 3, *E* and *F*) with the Bonferroni *post hoc* test and the 2-tailed Mann-Whitney test (Fig 3, *D*).

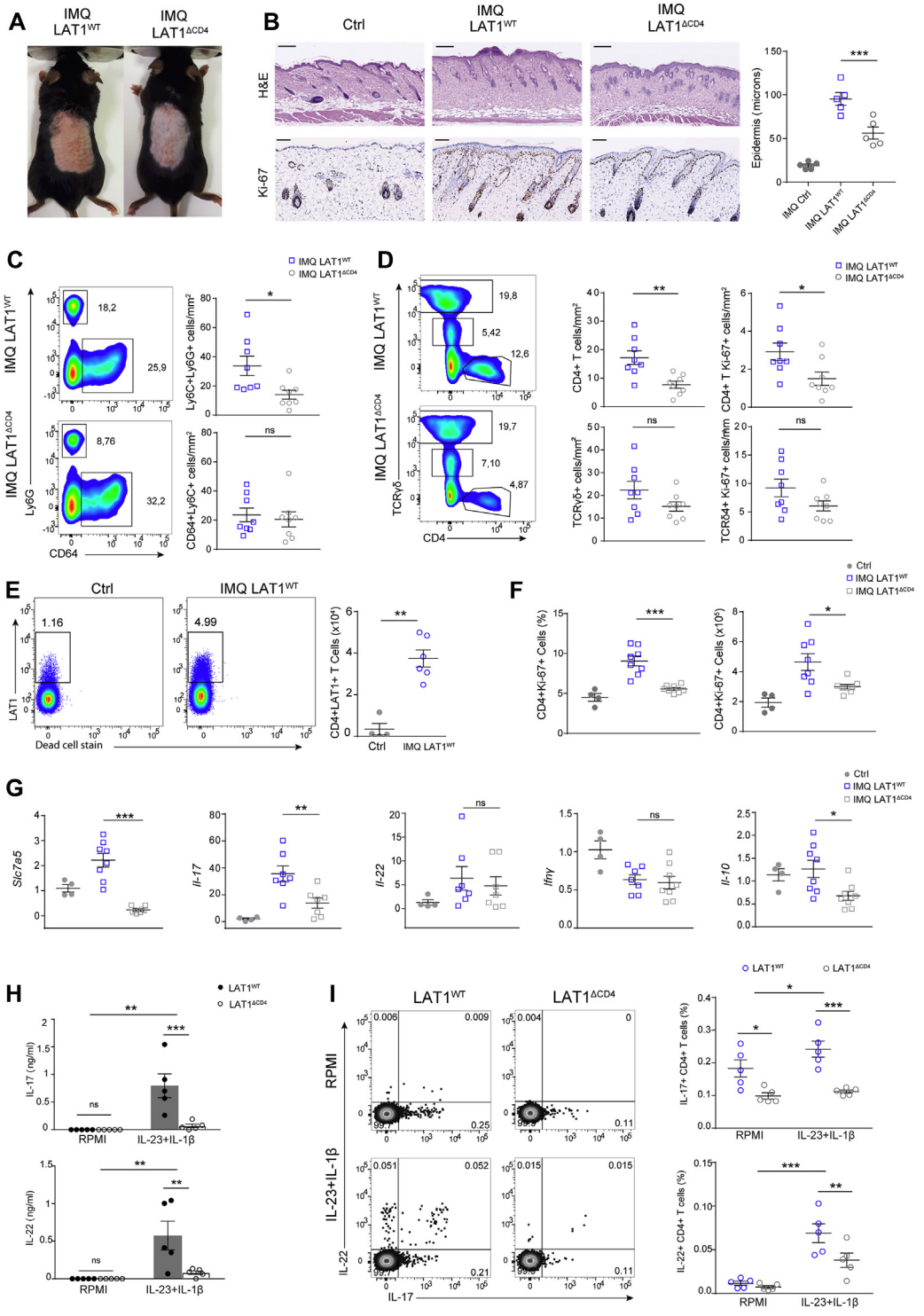

FIG 4. LAT1 expression in CD4 T cells contributes to development of IMQ-induced psoriasis. **A,** Representative pictures of IMQ-treated LAT1^{WT} and LAT1$^{\Delta CD4}$ mice. **B,** H&E-stained *(top)* and Ki-67–stained *(bottom)* sections. *Scale bars* = 100 μm. Averaged values of epidermal thickness are shown *(right)*. **C,** Skin neutrophil (Ly6G^{+}; *top*) and macrophage (CD64^{+}; *right*) quantification. **D,** Dermal CD4 (*right* and *bottom*) and γδ (*left* and *middle*) T cells and epidermal γδ T cells (*left* and *upper*) were analyzed. Total numbers *(left)* and Ki-67^{+} dermal CD4 and γδ T cells are shown *(right)*. **E,** LAT1 expression in CD4 T cells in lymph nodes. **F,** Frequencies *(left)* and absolute numbers *(right)* of CD4^{+}Ki-67^{+} cells in lymph nodes. **G,** Transcriptional expression in CD4 T cells after IMQ. **H** and **I,** Cytokines level (ELISA). **I,** Dot plots and frequencies of IL-17^{+} *(upper)* and IL-22^{+} *(lower)* CD4 T cells obtained from IMQ-treated mice and stimulated with IL-23 plus IL-1β and PMA/ionomycin. A pool of 2 independent experiments (Fig 4, *C*, *D*, *F*, and *G*) or data from one of 2 experiments (Fig 4, *E*, *H*, and *I*; n = 4-6) are shown. Data are shown as means ± SEMs. *ns*, Not significant. *$P < .05$, **$P < .01$, and ***$P < .001$, 2-tailed unpaired Student *t* test (Fig 4, *C-E*), 1-way ANOVA (Fig 4, *F* and *G*), or 2-way ANOVA (Fig 4, *H* and *I*) with the Bonferroni *post hoc* test.

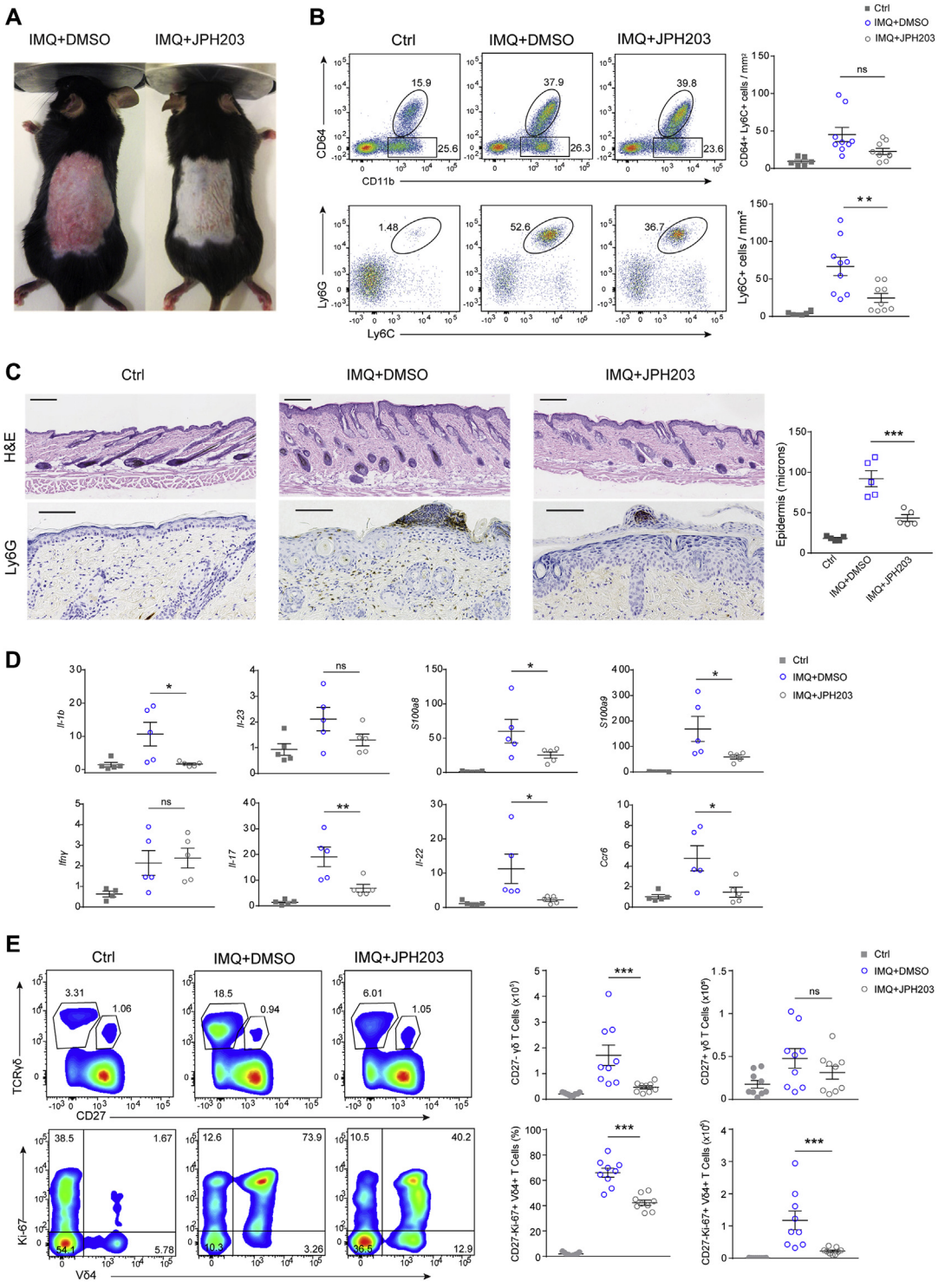

FIG 5. The inhibitor of LAT1, JPH203, prevents IMQ-induced skin inflammation. **A,** Representative pictures from mice treated with DMSO or JPH203 and topical application of IMQ. **B,** Dot plots identifying macrophages ($CD64^+CD11b^+$; *top*) and neutrophils ($Ly6G^+Ly6C^+$; *bottom*) in $CD45^+$ and $CD45^+CD64^-$ populations in the dorsal skin, respectively. Density values of macrophages and neutrophils are shown *(right)*. **C,** Representative H&E-stained *(top)* and Ly6G-stained *(bottom)* skin sections. *Scale bars* = 100 μm. Averaged values of epidermal thickness per mouse are shown in the graphic at right. **D,** Relative fold induction of indicated genes induced in the skin by IMQ. **E,** Representative density plots for $CD27^-$ and $CD27^+$ TCRγδ T cells in the $CD3^+$ gated population *(upper)* and frequencies of $V\delta4^+Ki\text{-}67^+$ cells on gated $CD27^-$ γδ T cells *(bottom)* in skin-draining lymph nodes. Absolute numbers or frequencies detected in cellular lymph node suspensions are shown per group *(right)*. A pool of 2 independent experiments (Fig 5, *B* and *E*) or data from one of 2 individual experiments (Fig 5, *D*) are shown (n = 4-5). Data are shown as means ± SEMs. *ns*, Not significant. $*P < .05$ and $**P < .01$, 1-way ANOVA with the Bonferroni *post hoc* test (Fig 5, *B*, *D*, and *E*).

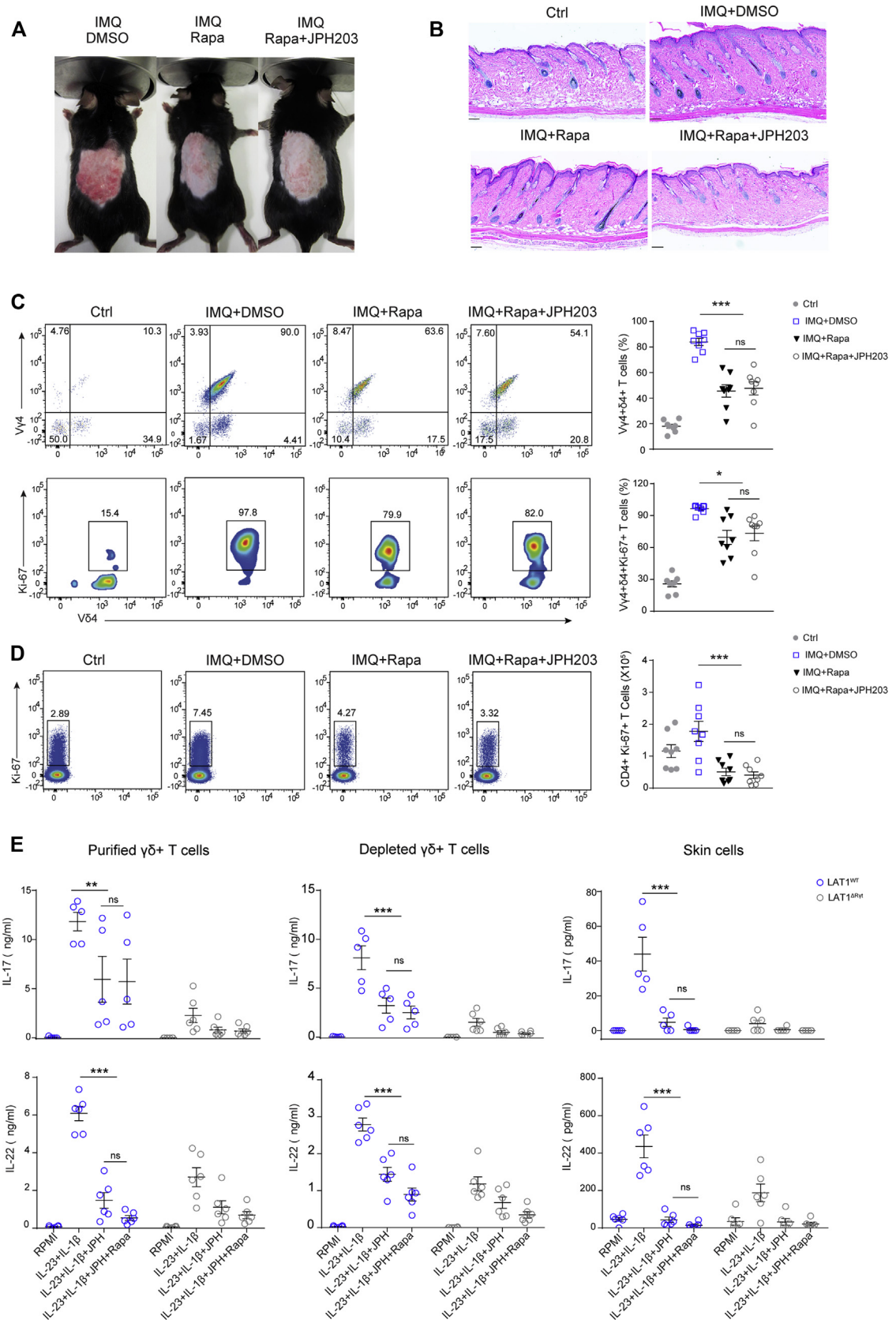

FIG 6. LAT1 acts as an upstream regulator of mTOR in the control of IMQ-induced psoriasis. **A,** Representative pictures of mice after 4 days of IMQ and treated with DMSO, rapamycin *(Rapa)*, or JPH203 plus rapamycin. **B,** Representative H&E-stained skin sections per group. *Scale bars* = 100 μm.

(see Fig E4, *F*). After 12 days in culture, stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin confirmed that LAT1 inhibition controls secretion of IL-17 and IFN-γ in human CD4 T cells. These results indicate that LAT1 expression is necessary for human and mouse CD4 T_H1 and T_H17 polarization.

Pharmacologic inhibition of LAT1 prevents IMQ-induced psoriasis in mice

Mice were randomly treated with dimethyl sulfoxide (DMSO; vehicle) or LAT1 inhibitor (JPH203) to ascertain whether pharmacologic inhibition of LAT1 can affect IMQ-induced psoriasis severity. Inhibition of LAT1 during IMQ application caused a marked reduction of the appearance of psoriasis hallmarks, such as redness, epidermal thickening, and neutrophil infiltration (Fig 5, *A-C*).

To confirm the beneficial effect of JPH203 against psoriasis, we evaluated transcriptional levels of several proinflammatory mediators in the skin (Fig 5, *D*) and draining lymph nodes (see Fig E5, *A*, in this article's Online Repository at www.jacionline.org). JPH203 treatment prevented the increase in IL-1β, IL-17A, IL-22, S100A8, and S100A9 levels induced by IMQ in the skin (Fig 5, *D*). Interestingly, reduced transcriptional expression of CCR6 was also detected in the skin of JPH203-treated mice compared with that in DMSO-treated mice, which is consistent with a reduction in skin infiltration by IL-17–releasing cells.[34] In contrast, we found no difference in IL-23 and IFN-γ levels (Fig 5, *D*). These experiments also revealed reduced transcription of IL-17 and IL-22 in skin-draining lymph nodes from JPH203-treated mice (see Fig E5, *A*). However, transcriptional levels of IL-10, IFN-γ, CCL5, and CCL20 were comparable in both the JPH203- and DMSO-treated groups. Likewise, increased transcriptional levels of the LAT1 gene *(Slc7a5)* but not the CD98 gene *(Slc3a2)* were found in the lymph nodes of IMQ-treated mice supplemented with JPH203 or DMSO compared with the control group (see Fig E5, *A*).

Inhibition of LAT1-mediated amino acid transport prevented IMQ-induced expansion of γδ T cells in skin-draining lymph nodes, which mostly express γ4 and δ4 chains (Fig 5, *E*). A significant reduction in numbers of $CD27^-$ γδ T cells was detected in mice treated with JPH203 compared with the DMSO group, whereas the population of $CD27^+$ γδ T cells remained unaffected (Fig 5, *E*). $CD27^-$ $γ4^+δ4^+$ T cells displayed high levels of Ki-67, indicating that they actively proliferate in the DMSO-treated group, whereas their proliferation was significantly reduced in mice treated with JPH203. These data indicate that LAT1 inhibition controls $CD27^-$ γδ T-cell proliferation (Fig 5, *E*).

Moreover, the skin of mice treated with JPH203 contained fewer infiltrating $Vγ4^+δ4^+$ T cells than the skin of DMSO-treated mice (see Fig E5, *B*). Skin-infiltrating $Vγ4^+δ4^+$ T cells were mostly positive for Ki-67 in DMSO-treated mice, indicating their proliferative activity. Also, JPH203 significantly reduced the frequency of Ki-$67^+Vγ4^+δ4^+$ T cells in the skin (see Fig E5, *B*). In addition to its effect on γδ T cells, JPH203 also controlled the expansion of CD4 T cells (data not shown).

In summary, systemic administration of the LAT1 inhibitor JPH203 attenuates the skin response to IMQ by limiting the expression of most proinflammatory mediators as well, as the proliferation of CD4 and γδ T cells.

Human γδ T cells can be activated and expanded by phosphorylated antigens, such as zoledronate.[35] After 10 days, the presence of Ki-67^+ γδ T cells is greater than 85% (data not shown), but cotreatment with JPH203 significantly prevented their expansion (see Fig E5, *C*). Western blot analysis of human γδ T cells confirmed expression of the LAT1-CD98 complex (see Fig E5, *C*).

The LAT1/mTOR axis controls inflammatory responses in the IMQ-psoriasis model

To evaluate the role of mTOR activation in the control of CD4 and $Vγ4^+δ4^+$ T-cell expansion in mice with LAT1 deletion or inhibition, we assessed the effect of the mTOR inhibitor rapamycin alone or in combination with JPH203 (JPH203 plus rapamycin) during IMQ challenge. Mice treated with rapamycin or JPH203 plus rapamycin displayed reduced skin inflammation induced by IMQ (Fig 6, *A*) and reduced epidermal thickness (Fig 6, *B*). Rapamycin and JPH203 plus rapamycin caused a similar reduction in the frequency of $Vγ4^+δ4^+$ T cells detected in the draining lymph nodes (Fig 6, *C*). The fraction of $Vγ4^+δ4^+$ T cells expressing Ki-67 was reduced by rapamycin, but no additional inhibitory effect was caused by JPH203 (Fig 6, *C*). In addition, expansion of CD4 T cells induced by IMQ was prevented by rapamycin or JPH203 plus rapamycin (Fig 6, *D*). Overall, no additional effect was observed by treatment with JPH203 in mice in which we had already inhibited mTOR signaling in inflammatory cells, indicating that LAT1 might be upstream of the mTOR pathway.

We next assessed the role of the LAT1/mTOR axis in the control of IL-17 and IL-22 secretion after stimulation with IL-23 and IL-1β. Lymph node cells from $LAT1^{ΔRγt}$ and $LAT1^{WT}$ mice treated with IMQ were used to obtain separate fractions of purified γδ T cells and the remaining lymph node cells. Both fractions, as well as total ear cell suspensions, were *in vitro* stimulated with IL-23 plus IL-1β with or without JPH203 alone or together with rapamycin (Fig 6, *E*). Cells derived from IMQ-treated $LAT1^{ΔRγt}$ mice barely responded to IL-23 plus IL-1β stimulation (ie, they did not secrete IL-17 and IL-22), and the inhibitory effects observed with JPH203 and JPH203 plus rapamycin were significant only in cells expressing LAT1. In addition, no significant differences were detected between cells treated with JPH203 alone and those treated with JPH203 plus rapamycin (Fig 6, *E*), further indicating that LAT1 acts upstream of mTOR to control IL-17–related cytokine release.

C, Dot plots of $Vγ4^+δ4^+$ *(upper)* and density plots of Ki-$67^+δ4^+$ *(bottom)* from live $CD27^-$ γδ T cells quantified in the lymph nodes. **D,** Dot plots of frequencies of Ki-67^+ cells from CD4 T cells in the lymph nodes. Individual values per mouse of frequencies (Fig 6, *C*) and total cell counts (Fig 6, *D*) are shown at right. **E,** Purified γδ T cells *(left)*, lymph node cells depleted of γδ T cells *(middle)*, and skin cell suspensions *(right)* of IMQ-treated mice of indicated genotypes were *in vitro* stimulated (24 hours) with IL-23 plus IL-1β and incubated with indicated inhibitors. Individual values per mouse of cytokine levels detected in supernatants by means of ELISA are shown. A pool of 2 independent experiments (Fig 6, *C* and *D*) or data from one representative experiment of 2 (Fig 6, *E*) are shown (n = 4-6). Data are shown as means ± SEMs. *ns*, Not significant. $*P < .05$, $**P < .01$, and $***P < .001$, 1-way ANOVA with the Bonferroni *post hoc* test (Fig 6, *C-E*).

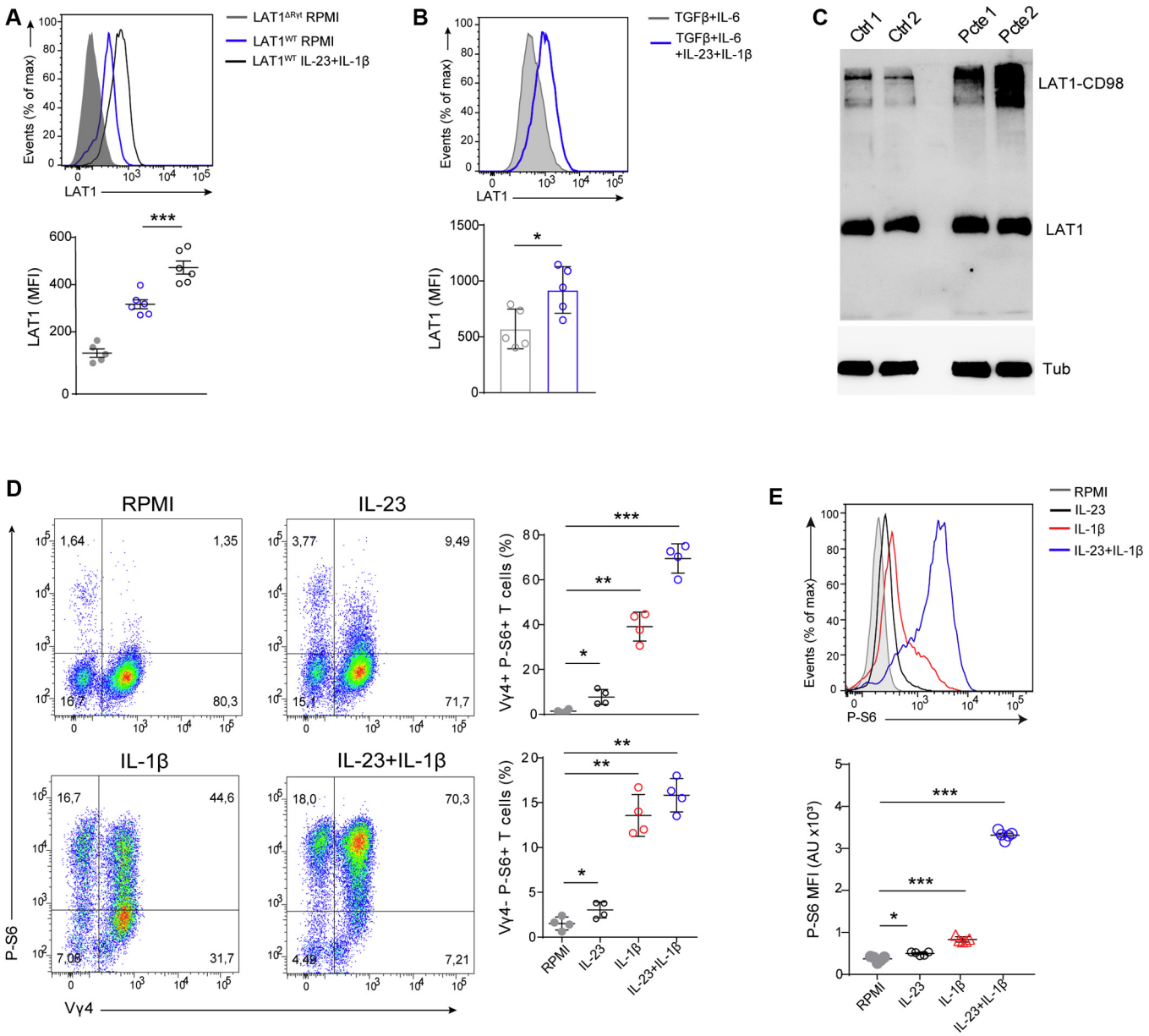

FIG 7. IL-23 and IL-1β upregulate LAT1 expression and induce mTOR activation in IL-17–secreting cells. **A** and **B,** Representative histograms *(upper)* and individual values *(bottom)* of LAT1 fluorescence in $V\gamma4^+$ cells from lymph node cells of IMQ-treated mice (Fig 7, *A*) and *in vitro*–skewed T_H17 cells from WT mice (Fig 7, *B*) after IL-23 plus IL-1β stimulation. **C,** Peripheral CD4 T cells from healthy donors and patients with psoriasis were *in vitro* stimulated (48 hours) with IL-23 plus IL-1β. Expression of LAT1 and the LAT1-CD98 heterodimeric complex determined by using Western blotting are shown. **D,** Total lymph node γδ T cells from IMQ-treated WT mice were stimulated (24 hours) with IL-23, IL-1β, or both. Dot plots *(left)* and frequencies *(right)* of P-S6 expression in $\gamma4^+$ *(upper)* and $\gamma4^-$ *(bottom)* cells are shown. **E,** Histograms *(upper)* and values of fluorescence intensity for P-S6 expression *(bottom)* in the total fraction of $CD27^-\gamma4^+$ T cells stimulated as in Fig 7, *D*. A representative experiment of 2 individual replicates is shown (at least n = 5 [Fig 7, *A, B, D,* and *E*] and n = 2 [Fig 7, *C*] samples per group). Data are represented as means ± SEMs. *ns*, Not significant. $*P < .05$ and $***P < .001$, 1-way ANOVA (Fig 7, *A, D,* and *E*) with the Bonferroni *post hoc* test and the 2-tailed paired Student *t* test (Fig 7, *B*).

LAT1 regulates the IL-23– and IL-1β–induced PI3K/AKT/mTOR pathway in IL-17–secreting cells

We further explored the mechanism through which LAT1 controls expansion of immune cells in psoriasis. A previous study proposed that AHR controls γδ T-cell proliferation in response to IMQ.[36] To address this, we inhibited LAT1 in $AHR^{+/-}$ and $AHR^{-/-}$ mice. JPH203 treatment dampened $CD27^-$ γδ and specifically $V\gamma4^+\delta4^+$ T-cell expansion in IMQ-treated $AHR^{+/-}$ and $AHR^{-/-}$ mice groups (see Fig E6, *A*, in this article's Online Repository at www.jacionline.org), indicating that the effect of LAT1 inhibition on γδ T-cell proliferation is largely independent of AHR expression. Moreover, JPH203 decreased the frequency and number of IL-17–secreting γδ T cells in both genotypes after IMQ challenge (see Fig E6, *B*).

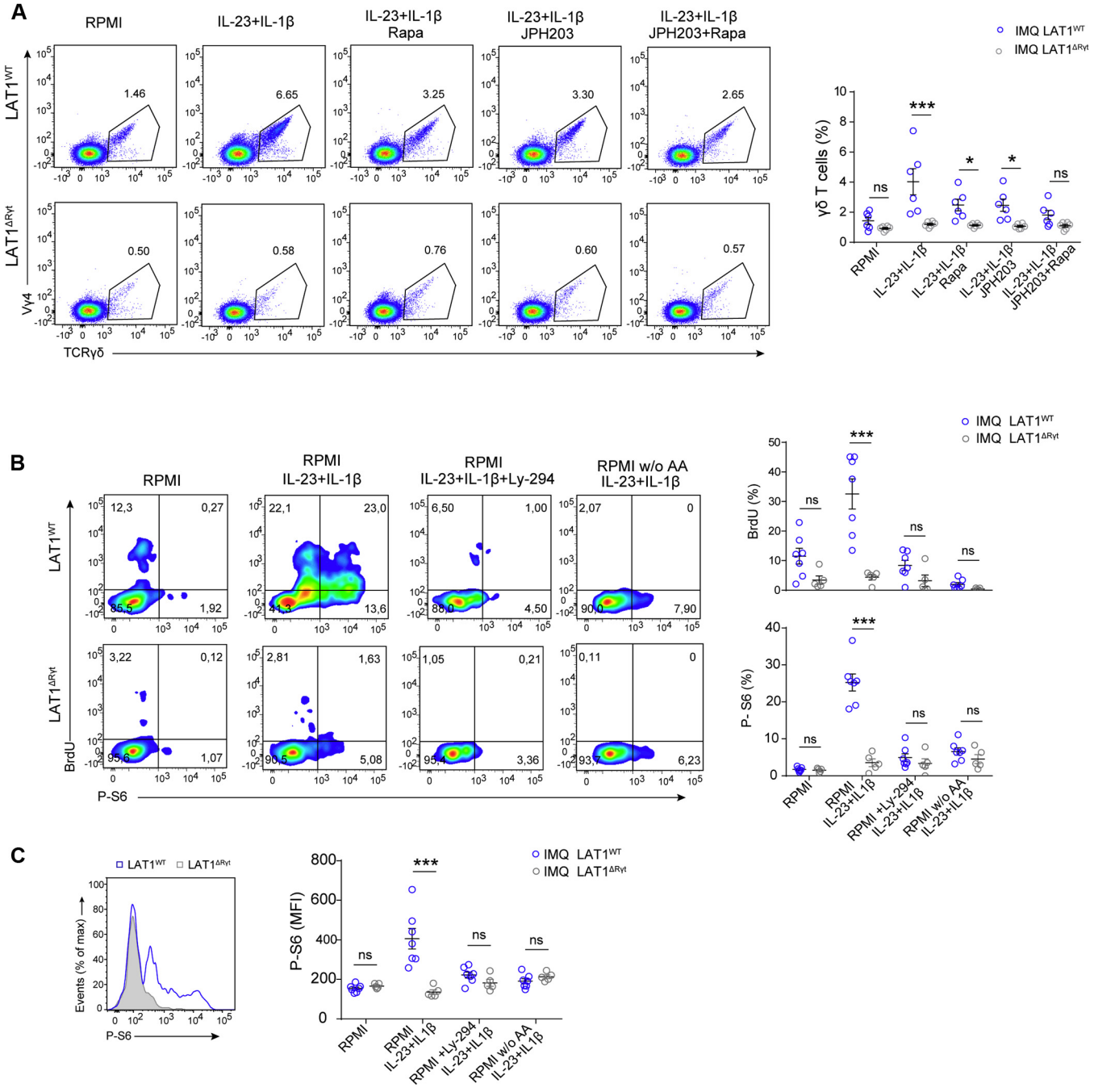

FIG 8. LAT1 is required to activate the PI3K/AKT/mTOR pathway induced by IL-23 and IL-1β to promote proliferation of IL-17–releasing cells. **A,** Lymph node cells from IMQ-treated mice were stimulated *in vitro* (48 hours) with the indicated cytokines and inhibitors. Dot plots *(left)* and individual values *(right)* of γδ T-cell frequencies from $CD3^+$ cells are shown. **B,** Density plots *(left)* and frequencies *(right)* of $BrdU^+$ and $P\text{-}S6^+V\gamma4^+$ cells obtained and stimulated as in Fig 7, *A*. **C,** Representative histogram of P-S6 expression *(left)* and individual values of P-S6 fluorescence *(right)* in $V\gamma4^+$ cells is shown. A representative experiment of 2 individual replicates is shown (at least n = 5). Data are shown as means ± SEMs. *ns*, Not significant. $*P < .05$ and $***P < .001$, 2-way ANOVA with the Bonferroni *post hoc* test.

To characterize the molecular triggers of LAT1 expression in psoriasis, we assessed the effect of the proinflammatory cytokines IL-23 and IL-1β. Both cytokines play crucial roles in T_H17 development[37] and promote extrathymic commitment of $IL\text{-}17^+$ γδ T cells in the IMQ model.[12] LAT1 expression was significantly increased by IL-23 plus IL-1β stimulation in $V\gamma4^+$ T cells from the lymph nodes of mice treated with IMQ (Fig 7, *A*). Stimulation with IL-23 and IL-1β also increased LAT1 expression of $CD4^+IL\text{-}17^+$ T cells differentiated *in vitro* (Fig 7, *B*). Moreover, peripheral CD4 T cells from patients with psoriasis expressed

high levels of LAT1-CD98 complex when stimulated with IL-23 plus IL-1β compared with cells from healthy donors (Fig 7, *C*). These results suggested that LAT1 is essential for the expansion of IL-17–secreting T lymphocytes induced by IL-23 and IL-1β.

IL-23 and IL-1β also induce activation of the mTOR pathway, as assessed by an increase in P-S6 detection, in γδ T cells (Fig 7, *D* and *E*). Remarkably, the combination of IL-23 plus IL-1β potentiates the effect of each cytokine alone on P-S6 activation in both $\gamma4^+$ and $\gamma4^-$ T cells (Fig 7, *D* and *E*).

Stimulation with IL-23 plus IL-1β induced expansion of $V\gamma4^+$ cells but only when LAT1 was expressed (Fig 8, *A*). However, expansion of $V\gamma4^+$ T cells from $LAT1^{WT}$ mice is reduced by addition of either mTOR or LAT1 inhibitors. These inhibitors had no effect on cells from $LAT1^{\Delta R\gamma t}$ mice (Fig 8, *A*). Finally, the combination of mTOR and LAT1 inhibitors had no additive effect, further confirming that LAT1 and mTOR are likely part of the same signaling pathway in these cells. The effects of IL-23 plus IL-1β stimulation in BrdU incorporation and S6 phosphorylation in $V\gamma4^+$ T cells were completely blocked by PI3K inhibitor (Ly294002) or by amino acid depletion (Fig 8, *B*). Cells from $LAT1^{\Delta R\gamma t}$ mice did not display mTOR activation or proliferation under any of the conditions assayed (Fig 8, *C*). These data indicate that IL-23 and IL-1β trigger LAT1 expression in $V\gamma4^+$ T cells and T_H17 cells, which promotes PI3K/AKT-induced mTOR signaling to control their expansion and IL-17 secretion.

DISCUSSION

The pathogenesis of psoriasis requires complex mechanisms that involve the interplay between keratinocytes and inflammatory cells.[1] Genetic studies aimed at identifying new genetic pathways associated with psoriasis risk demonstrated that alterations in metabolic pathways, such as those involved in transporting inorganic ions and amino acids, positively correlate with increased psoriasis risk.[38] Increased transcriptional levels of *Slc7a5* were detected in skin samples of patients with psoriasis.[22] The present study represents the first description of the increased expression of LAT1 protein in psoriatic lesions in keratinocytes and infiltrating lymphocytes. Pharmacologic inhibition of LAT1 effectively blocks skin inflammation induced in mice by means of IMQ application. The anti-inflammatory effects of the inhibitor can be recapitulated by genetic deletion of LAT1 in lymphocytes, including RORγt-expressing cells, such as γδ T cells and CD4 T cells.

The mTOR pathway is hyperactivated in keratinocytes from patients with psoriasis.[39] Its aberrant induction is mediated by proinflammatory cytokines, such as IL-1β, IL-17A, and TNF-α, increasing proliferation and reducing expression of differentiation markers.[40] Our results indicate that LAT1 expression in keratinocytes is not essential for control of keratinocyte proliferation, indicating a functional compensation with alternative amino acid transporters, such as LAT2 or LAT3, which are also expressed in the epidermal layer in patients with psoriasis.

A major feature of the IMQ model is expansion of $CD27^-\gamma4^+\delta4^+$ T cells in draining lymph nodes that produce IL-17.[12,28,41] A similar expansion of this population is observed in a mouse experimental autoimmune encephalomyelitis model.[42] Our results demonstrate that $IL\text{-}17^+\gamma4^+\delta4^+$ T-cell expansion after skin sensitization with IMQ is blocked by targeting LAT1 pharmacologically or genetically. Importantly, LAT1 inhibition also blocks expansion of human γδ T cells. Further experiments will address whether LAT1 deletion controls $\gamma4^+$ T-cell expansion in patients with other IL-17–mediated diseases, such as autoimmune encephalomyelitis.

LAT1 inhibition controls the proliferation of $V\gamma4^+\delta4^+$ T cells, even in the absence of AHR. However, LAT1 inhibition in the presence of the canonical mTOR inhibitor rapamycin had no additive effect. Inhibition of mTOR by targeting LAT1 could be an alternative approach to specifically target immune cells stimulated by IL-23 and IL-1β in patients with inflammatory diseases.

LAT1 deletion in dermal γδ T cells also effectively controls IL-23–induced skin inflammation. Nevertheless, the main disadvantage of IMQ- and IL-23–induced murine models of psoriasis is their dependence on γδ T cells.[8,10] Although the increased frequency of γδ T cells has been observed also in human psoriatic lesions,[2] their relevance in the onset or recurrence of disease is not clearly established.[43,44] Indeed, human psoriatic lesions show a greater frequency of IL-17–releasing $TCR\alpha\beta^+$ T cells than γδ T cells.[32] Our data demonstrate that LAT1 deletion in CD4 T cells dampens IMQ-induced skin inflammation. Moreover, LAT1 inhibition successfully impairs the T_H17 differentiation program in human CD4 T cells. Because the LAT1 inhibitor JPH203 is currently in clinical trials,[21] we propose that blocking LAT1-mediated amino acid transporter merits consideration as a novel immunosuppressive strategy to control the expansion of innate and adaptive T cells in the reactive state in patients with inflammatory diseases.

Whether mTOR activation is required for T_H17 cell expansion has been studied extensively[45]; however, the role of T_H17 driver cytokines, such as IL-23 and IL-1β, in mTOR activation had not been fully characterized. T_H17 cell development required TCR-mediated activation, which triggers the PI3K/AKT/mTOR signaling axis. LAT1 expression is induced after TCR engagement and is essential to properly activate αβ T cells.[17] LAT1 expression after TCR activation requires nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) and activator protein 1 activation.[46] The data herein clearly show that IL-23 and IL-1β stimulation also regulates LAT1 expression in T_H17 cells, as well as in γδ T cells, which do not receive TCR input.

IL-23–induced Janus kinase 2 activation triggers the PI3K/AKT and NF-κB pathways.[47] IL-1β stimulation can induce NF-κβ activation through myeloid differentiation response gene–88,[48] which is also essential for mTOR activation in T_H17 cells.[49] Moreover, IL-1R signaling induced PI3K/AKT phosphorylation and mTOR activation to promote differentiation of pathogenic T_H17 cells.[50] Our data indicate that inhibition of PI3K/AKT or amino acid depletion abrogated mTOR activation induced by IL-23 and IL-1β in γδ T cells. Importantly, PI3K/AKT signaling is important for expansion of IL-17–secreting γδ T cells.[51,52] Our data suggest that IL-23 and IL-1β induce LAT1 expression as a positive feedback loop to drive activation of that PI3K/AKT/mTOR pathway, which is essential for increased survival and expansion of IL-17–releasing cells.

LAT1 inhibition decreased transcriptional levels of IL-1β in the skin. This effect is potentially related to blockade of amino acid uptake in skin macrophages, which is also involved in IL-1β secretion.[53] This additional anti-inflammatory effect of JPH203 might contribute to prevention of psoriasis. However, genetic deletion of LAT1 in γδ and CD4 T cells was sufficient to control disease development.

This work provides the first evidence that murine psoriasis models can be precisely modulated by targeting inflammatory cell metabolism. Our data postulate that the LAT1 inhibitor JPH203, which has already been tested in patients with cancer for biosafety,[54] could be an alternative to control chronic skin inflammation.

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Key messages

- **LAT1 (SLC7A5) expression is increased in patients with psoriasis in both keratinocytes and dermal infiltrating lymphocytes, and its expression is upregulated by IL-23 and IL-1β.**
- **LAT1 inhibition does not affect keratinocyte proliferation but impairs expansion of IL-17–secreting γδ and CD4 T cells.**
- **LAT1 controls IL-23 plus IL-1β–induced PI3K/AKT/mTOR signaling in IL-17–secreting γδ and CD4 T cells to guarantee their expansion and cytokine secretion.**

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METHODS

Chemical reagents

The LAT1-specific inhibitor JPH203, (S)-2-amino-3-(4-((5-amino-2-phenylbenzo [d] oxazol-7-yl) methoxy)-3,5-dichlorophenyl) propanoic acid, was purchased from MedKoo Biosciences (Morrisville, NC). All other reagents were purchased from Sigma (St Louis, Mo), unless otherwise indicated.

Mice

Mice were bred and maintained in the specific pathogen–free animal facilities of Centro Nacional de Investigaciones Cardiovasculares (Madrid, Spain). All animal experiments were performed in accordance with protocols approved by the institutional animal care committee and were approved by local and European ethics committees. For all experiments, we used littermates derived from crossing Cre-negative Slc7a5$^{fl/fl}$ tomato$^{fl/wt}$ female mice with Cre-positive Slc7a5$^{fl/wt}$ tomato$^{fl/wt}$ male mice. LAT1^{WT} mice are Cre$^{+/-}$Slc7a5$^{fl/wt}$Tomato$^{fl/wt}$ mice, and LAT1$^{\Delta}$ mice are Cre$^{+/-}$Slc7a5$^{fl/fl}$Tomato$^{fl/wt}$ mice. K5-CreERT2 mice were kindly provided by Erwin Wagner (Medical University, Vienna, Austria). Mice (4 weeks) were fed with a 400-ppm tamoxifen diet (TD55125; Envigo, Indianapolis, Ind) for induction of Cre protein in the skin. AHR$^{-/-}$ mice were kindly provided by Pedro Salguero (Universidad de Extremadura, Badajoz, Spain). AHR$^{-/-}$ mice and their control littermate AHR$^{+/-}$ mice were obtained by breeding heterozygous parents. Forkhead box P3–mRFP reporter mice backcrossed with IL-17–green fluorescent protein reporter mice were kindly provided by Richard A. Flavell (Yale University, New Haven, Conn) and used as normal (WT) mice for experiments with either JPH203 or rapamycin inhibitors. All mouse strains used are on the C57BL/6 background. Experiments were conducted with male mice (8-12 weeks) kept on a regular 12-hour light/dark cycle (7 AM-7 PM light period), with food and water available *ad libitum*.

Human subjects

Patients with moderate-to-severe psoriasis who were recruited for the study had a Psoriasis Area and Severity Index of 8.0 or greater and washout periods of at least 14 days for topical corticosteroids and any systemic therapy. Skin punch biopsy specimens (3 mm) were obtained from patients with lesion plaque–type psoriasis and healthy volunteers. Blood samples (10 mL) were also collected from patients with psoriasis and healthy volunteers. The study was approved by the Hospital Universitario de La Princesa ethics committee, and all participants provided written informed consent.

Psoriasis model induction and treatment

Induction of local psoriasis-like inflammation on ear skin was done through daily topical administration of 10 mg of IMQ cream (5%) in each ear for 4 or 5 days. For systemic psoriasis induction, mice were mostly treated daily on shaved and depilated back skin with 50 mg of IMQ cream for 4 or 5 days. Exceptionally, LAT1$^{\Delta CD4}$ mice received 20 mg of IMQ daily on back skin for 8 days. When indicated, mice received daily doses of JPH203 (50 mg/kg of body weight) and rapamycin (5 mg/kg of body weight) dissolved in DMSO (through the intraperitoneal route) during the course of IMQ treatment. Mice without IMQ or treatment application are always included as a control group. The IL-23 model of psoriasis was conducted, as previously described.[E1] At least 10 intradermal injections of recombinant mouse IL-23 (500 ng in 20 μL of PBS; eBioscience, San Diego, Calif) were performed per mouse on alternate days. When indicated, mice received an injection of 50 μL (250 μg) of Brefeldin A (Sigma) dissolved in ethanol (5 mg/mL) and administered intraperitoneally 12 hours before death. For *in vivo* BrdU labeling of cells, 1 mg of BrdU dissolved in 100 μL of PBS was injected intraperitoneally 3 hours before mice were killed.

Skin analysis and staining

For histologic analysis, paraformaldehyde-fixed, paraffin-embedded dorsal skin sections were prepared and stained with H&E. At least 3 skin sections (3-5 μm) 300 μm apart from each other were analyzed per mouse. For quantification of epidermal thickness, at least 10 measurements, randomly performed between all sections, were averaged per mouse. For immunohistochemical staining, skin sections were deparaffinized, boiled in the suggested antigen retrieval solution, and incubated with the primary antibodies indicated in Table E1. Slides were developed with diaminobenzidene substrate (K3468; Dako, Glostrup, Denmark) and then counterstained with Mayer hematoxylin. For immunofluorescence of LAT1 in mice, fresh skin fragments were embedded in OCT compound. IL-17–green fluorescent protein was detectable in unfixed skin sections without staining.

Human skin sections were deparaffinized, boiled in the suggested antigen retrieval solution, blocked with BSA solution (2%), and incubated with the primary antibodies indicated in Table E1 for 18 hours at 4°C. The secondary antibodies used were the EnVision FLEX system for immunohistochemistry detection of LAT1/LAT2 (Dako) and Alexa Fluor 647–labeled chicken anti-rabbit for LAT1/LAT2 and LAT3 immunofluorescences. Nuclei were counterstained with 4′-6-diamidino-2-phenylindole dihydrochloride. Pictures were taken with a Zeiss LSM confocal microscope (Zeiss, Oberkochen, Germany) and analyzed with LSM image browser software.

Lymphocytes and skin cell preparations for FC

Tissues were dissected and grated through a nylon mesh (70-μm; BD Biosciences, San Jose, Calif) to obtain single-cell suspensions from peripheral lymph nodes. RBC lysis was performed with BD Pharm Lyse Buffer (BD Biosciences). Ears were split into dorsal and ventral halves, and dorsal skin was cut into small pieces. Tissues were digested for 30 minutes at 37°C in RPMI containing penicillin-streptomycin, HEPES buffer, 83 μg/mL Liberase (Roche Applied Science, Mannheim, Germany), 100 μg/mL DNAse I (Sigma), and 0.5 mg/mL Collagenase IV (Sigma) under constant stirring. Digestion enzymes were quenched by addition of 5 mmol/L EDTA and 0.5% BSA. Undigested tissue was homogenously disaggregated with 7-mm stainless steel beads (Life Technologies, Grand Island, NY) in a TissueLyser LT (Qiagen, Hilden, Germany), one 3-minute cycle (20 osc/sec). Isolated skin cells were flowed through a 70-μm nylon filter (BD Biosciences). For analysis of keratinocytes, dorsal skin from mice treated or not with IMQ was incubated with 4 U/mL Dispase II (Roche) in MEM medium by 24 hours at 4°C. True count beads (BD Biosciences) were added to cell suspensions to quantify the total number of cells in further FC.

FC

Single-cell suspensions were incubated with Fc-blocking antibodies and subsequently stained with 1:200 dilutions of the appropriated surface marker antibodies detailed in Table E1. The staining panels always included dead cell staining with Fixable Yellow Viability Dye (Molecular Probes, Eugene, Ore). BrdU detection was performed according to the manufacturer's protocol (BD PharMingen). P-S6 (Ser235/236) (pS6) and Ki-67 staining were conducted with the FOXP3 Transcription Factor Staining Buffer Set (eBioscience) and with directed labeled antibodies against Ki-67 (B56; BD Biosciences) and pS6 (D57.2.2E; Cell Signaling, Danvers, Mass). For LAT1 and LAT2 detection, cells were fixed and permeabilized with Cytofix/Cytoperm Kit (BD Biosciences), and Alexa Fluor 647–labeled chicken anti-rabbit was used as a secondary antibody. A customized rabbit anti-mouse LAT2 (kindly provided by Dr Manuel Palacín, IRB, Barcelona, Spain) was used. Further description of antibodies is included in Table E1.

After staining, cells were washed and analyzed with FACSCanto or LSRFortessa (BD Biosciences). Data were further analyzed with FlowJo10 software (TreeStar, Ashland, Ore).

In vitro cultures and cytokine detection

Mouse cells were cultured in RPMI or Iscove modified Dulbecco medium supplemented with FCS (5%), 25 mmol/L HEPES, antibiotics, sodium pyruvate, and β-mercaptoethanol. When indicated, cells were incubated in

RPMI 1640 medium without amino acids and supplemented with normal FCS (5%; United States Biological, Salem, Mass). Naive CD4 T cells were purified from lymph nodes by using commercial kits (STEMCELL Technologies, Vancouver, British Columbia, Canada).

Naive CD4 T cells were seeded (1×10^6 cells/mL) in a 24-well plate coated with 5 μg/mL anti-CD3 (145 2C11; Tonbo Biosciences, San Diego, Calif) for 48 hours to test LAT1 expression and amino acid uptake experiments. For *in vitro* differentiation studies, naive CD4 T cells were seeded (1×10^6 cells/mL) in supplemented Iscove modified Dulbecco medium in 24-well plates coated with 5 μg/mL anti-CD3 (145 2C11) and 1 μg/mL anti-CD28 (37.51) antibodies (Tonbo Biosciences) and steered toward the T_H17 lineage with IL-6 (50 ng/mL), IL-23 (10 ng/mL), IL-1β (10 ng/mL), and TGF-β (1 ng/mL) for 4 days. When indicated, the T_H17 cells were cultured with IL-6 and TGF-β for 4 days at concentrations indicated previously, and stimulation with IL-23 and IL-1β was tested only by 24 hours.

Total CD4 T cells purified from $LAT1^{WT}$ and $LAT1^{\Delta CD4}$ mice after IMQ application were incubated by 18 to 24 hours with IL-23 and IL-1β cytokines (10 ng/mL each) to stimulate secretion of cytokines and P-S6 induction or 48 hours to assess proliferation. Similarly, cell suspensions from lymph nodes of $LAT1^{WT}$ and $LAT1^{\Delta R\gamma t}$ mice after IMQ application were split into 2 fractions: one was subjected to γδ T-cell depletion using anti-TCRγδ (clone GL3), and the other was used to purify γδ T cells with the EasySep Mouse Selection Kit (STEMCELL Technologies). The resulting fractions (purified γδ and γδ depleted) were *in vitro* stimulated by 18 to 24 hours with IL-23 and IL-1β to assess cytokine release by means of ELISA. Ear cell suspensions after IMQ application were also *in vitro* stimulated with IL-23 and IL-1β to assess cytokine release. Supernatants were collected, and IL-17 and IL-22 levels were assessed by using mouse ELISA Ready-SET-Go Kits (Fisher Scientific, Waltham, Mass). CD4 T cells were further stimulated with PMA (50 ng/mL; Sigma), ionomycin (1 mg/mL; Sigma-Aldrich), and brefeldin A (GolgiStop, 1 μg/mL; BD Biosciences) at 37°C in a 10% CO_2 atmosphere for 4 hours. After staining of surface markers, cells were fixed and permeabilized (Cytofix/Cytoperm and Perm/Wash Buffer; BD Biosciences), followed by staining with mAbs to mouse IL-17A and IL-22 (eBioscience). True count beads (BD Biosciences) were added to *in vitro* cultures to quantify the total number of expanded γδ T cells. Rapamycin (1 μmol/L), LY294002 (10 μmol/L), and JPH203 (10 μmol/L) inhibitors were added to the cultures when indicated. When indicated, BrdU at a final concentration of 5 μmol/L was added to assess proliferation according to the instructions of the BrdU Flow Kits (BD Life Science-Bioscience).

PBMCs from healthy donors were obtained, and CD4 T cells were isolated by using the EasySep Human CD4 T Cell Isolation Kit (STEMCELL Technologies). For T_H17 differentiation, isolated CD4 T cells were cultured for 12 days in RPMI 1640 medium supplemented with FCS (5%), 25 mmol/L HEPES, antibiotics, and sodium pyruvate with anti-CD3 (5 μg/mL; catalog no. 300314, RRID:AB314050; BioLegend) plus anti-CD28 mAbs (2 μg/mL; catalog no. 555725; BD). The following combination of cytokines and blocking antibodies was appropriate for T_H17 polarization: rhIL-6 and IL-1β (10 ng/mL), rhIL-23 (20 ng/mL), rhTGF-β1 (2 ng/mL), anti–IFN-γ (10 μg/mL), and anti–IL-4 (10 μg/mL; all cytokines from R&D Systems, Minneapolis, Minn) were added each 48 hours. JPH203 at 10 μmol/L or DMSO was also added to the culture on alternate days. At day 12, cells were further stimulated by 4 hours with PMA (50 ng/mL; Sigma) and ionomycin (1 mg/mL; Sigma-Aldrich) in the presence of brefeldin A (GolgiStop, 1 μg/mL; BD Biosciences). Fixed and permeabilized cells (Cytofix/Cytoperm and Perm/Wash Buffer; BD Biosciences) were stained with anti–IL-17 (BL168) and anti–IFN-γ (B27) antibodies (BioLegend) and analyzed in a FACScanto cytometer.

For expansion of human γδ T cells, total human PBMCs (10^6 cells/mL) from heathy donors (buffy coats) were stimulated in a 24-well plate in culture medium EX-VIVO 15 (Lonza, Bornem, Belgium) and expanded with zoledronic acid (5 μmol/L), as previously reported.[E2] At day 2, IL-2 (100 U/mL) and JPH203 inhibitor (10 μmol/L) were added and then replaced every 48 hours. At day 10, the number of Ki-67^+CD27^- γδ T cells was analyzed in a FACSCanto cytometer. LAT1 expression associated with CD98 was also confirmed in expanded (10 days) human γδ T cells by means of Western blotting.

RNA extraction and quantitative PCR analysis

Tissue total RNA was isolated with TRI Reagent (Sigma) or the Qiagen RNeasy Kit (Qiagen). Residual DNA contamination was removed with the Turbo DNA-free Kit (Ambion, Thermo Fisher, Waltham, Mass). Total RNA (200-1000 ng) was reverse transcribed to cDNA with a Reverse Transcription Kit (Applied Biosystems, Foster City, Calif). Quantitative PCR was then performed in an AB7900_384 (Applied Biosystems) by using SYBR Green (Applied Biosystems) as a reporter. Gene-specific primers used are listed in Table E2. Expression of each gene of interest was normalized to at least 2 housekeeping genes: β-actin *(ACTB)* and glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)*. Data are presented as averaged relative fold differences calculated by using the $2^{-\Delta\Delta Ct}$ method with average values of healthy mice as a reference.

Western blotting

CD4 T cells from $LAT1^{\Delta R\gamma t}$ and $LAT1^{\Delta CD4}$ mouse cell lines were cultured (1×10^6 cells/mL) in RPMI medium for 24 hours in the presence of anti-CD3 (5 μg/mL). After lysis with RIPA buffer supplemented with protease and phosphatase inhibitor cocktails (Roche), lysates were separated by using SDS-PAGE and immunoblotted with anti-LAT1 antiserum (kindly provided by Dr P. Taylor, Dundee, United Kingdom).[E3] The loading control was carried out with a rabbit anti-mouse SMC protein 1A antibody (A300-055A; Bethyl Laboratories, Montgomery, Tex).

γδ T cells from PBMCs of patients with psoriasis were purified with the EasySep Human Gamma Delta T Cell Isolation Kit (STEMCELL Technologies) after 7-10 days in culture with zoledronate. CD4 T cells purified from PBMCs of patients with psoriasis or healthy volunteers were stimulated with anti-CD3 (2 μg/mL), anti-CD28 (1 μg/mL), IL-23 (20 ng/mL), and IL-1β (20 ng/mL) for 48 hours. Lysates of human γδ T cells and CD4 T cells were done with RIPA buffer, separated by using SDS-PAGE, and immunoblotted with rabbit anti-LAT1 (5347S; Cell Signaling Technology) and mouse anti–β-actin (47778; Santa Cruz Biotechnology, Dallas, Tex) or mouse anti–α-tubulin (T6199; Sigma-Aldrich) as a loading control. Detection of LAT1, LAT2, and CD98 in cell lines (HaCaT, Caco-2, HeLa, and J77) was also conducted in fresh lysates prepared with supplemented RIPA buffer and with the antibodies indicated in Table E1. All primary antibodies were detected with horseradish peroxidase–conjugated goat anti-rabbit (Pierce, Rockford, Ill). Protein bands were analyzed with the LAS-3000 CCD system and Image Gauge 3.4 software (Fuji Photo Film, Tokyo, Japan).

Quantitative profile of L-Leu by using liquid chromatography–tandem mass spectrometry

Serum L-Leu profiles of normal (WT) and immunocompromised *($Rag1^{-/-}$)* mice treated or not with IMQ (for 5 days, 50 mg/d) were measured by using liquid chromatography–tandem mass spectrometry. Serum samples were obtained from at least 20 animals per genotype (10 healthy control mice and 10 mice with psoriasis) after 60 minutes of coagulation at 4°C and were immediately stored at −80°C until use. Stock solutions of leucine (Sigma) and ^{13}C11-Trp (Cambridge Isotope Laboratories, Tewksbury, Mass) were prepared in water (LC/MS grade) at 1000 ppm and used as an external standard and an internal standard, respectively.

Amino acid uptake assays

Naive CD4 T cells obtained from $LAT1^{WT}$, $LAT1^{\Delta R\gamma t}$, and $LAT1^{\Delta CD4}$ mice were cultured (1×10^6 cells/mL) in RPMI medium for 24 hours in the presence of anti-CD3 (5 μg/mL) and used to test amino acid uptake in the presence of LAT1 inhibitors. The ^{3}H-radiolabeled amino acids L-phenylalanine and L-Leu (PerkinElmer, Waltham, Mass) were added (0.5 μCi/ml) in HBSS (Gibco, Carlsbad, Calif) at a final extracellular L-Leu concentration of 5 μmol/L. Amino acid uptake was measured at 60 minutes at 37°C. Incubation with the LAT1 inhibitors JPH203

(10 μmol/L) and 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (40 mmol/L) was done 10 minutes before addition of radioactivity. Uptake was stopped by addition of 20 mmol/L cold L-Leu to quench L-System. At the end of the assay period, cells were harvested onto glass-fiber filters using a Tomtec 96-well parallel harvester (Tomtec, Hamden, Conn). B-radioactivity was counted in a Beckman LS 6500 Multi-Purpose Scintillation Counter (Beckman Coulter, Fullerton, Calif). At least 6 replicates were assessed for each data point.

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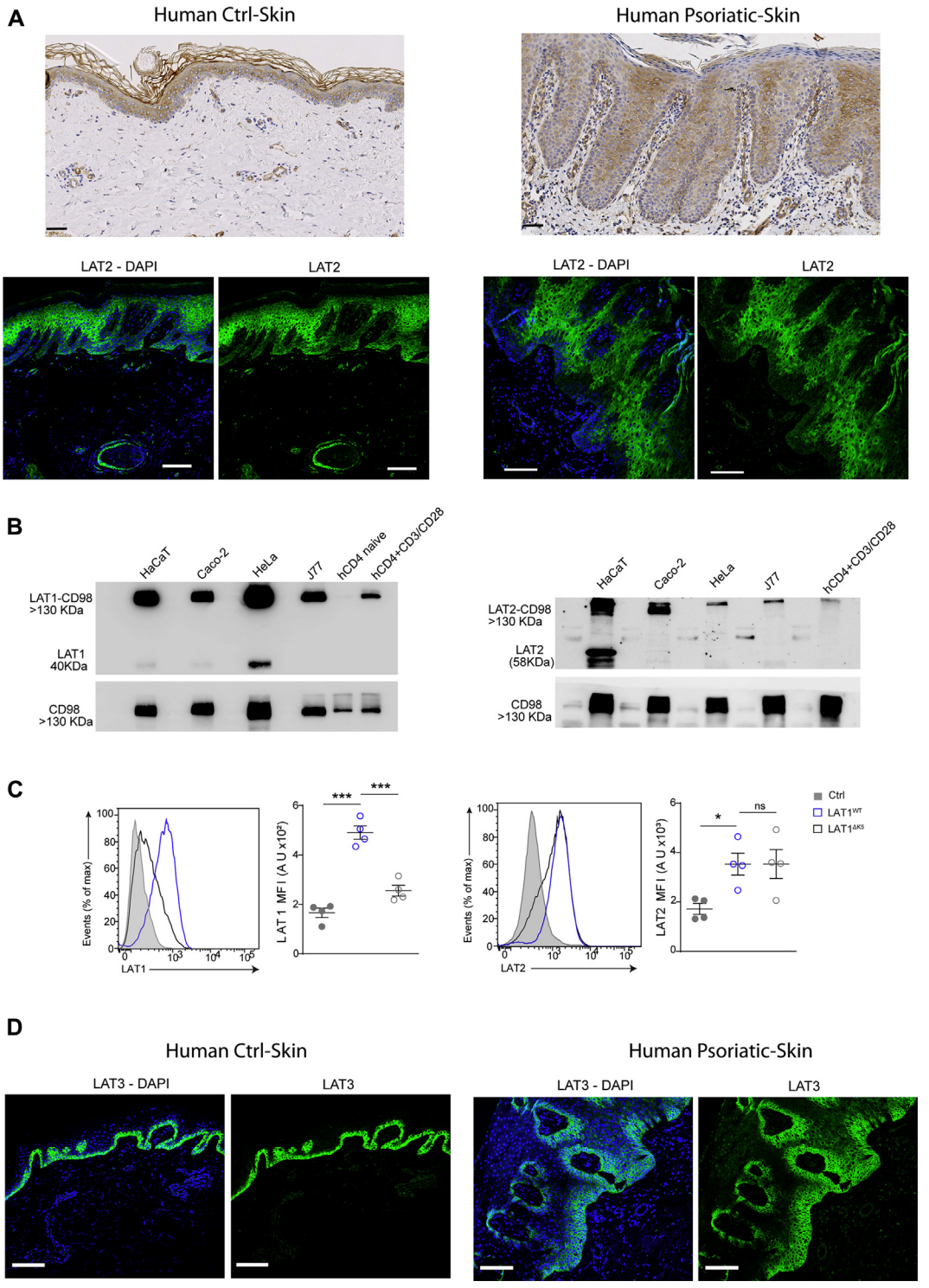

FIG E1. LAT2 and LAT3 expression is detected in the epidermal layer in patients with psoriasis. **A,** LAT2 detection by means of immunohistochemistry *(upper)* and immunofluorescence *(bottom)* in skin biopsy specimens from healthy donors *(left)* and patients with psoriasis *(right)*. **B,** LAT1, LAT2, and CD98 detection by means of Western blotting in nonreduced conditions in 10 μg of total protein from different types of cell lines and primary human CD4 T cells (both naive and activated). **C,** Histograms and values of mean fluorescence intensity of LAT1 *(left)* and LAT2 *(right)* expression in $LAT1^{\Delta K5}$ and $LAT1^{WT}$ keratinocytes after IMQ application. **D,** LAT3 detection by means of immunofluorescence in human skin samples. LAT2 and LAT3 signals are shown in green, and nuclei were stained with 4′-6-diamidino-2-phenylindole dihydrochloride (*DAPI*; blue). *Scale bars* = 100 μm. At least 3 human skin samples of each condition were simultaneously analyzed in each study. A representative experiment of at least 2 individual replicates is shown (n = 4-5; Fig E1, *C*). Data are shown as means ± SEM. *ns*, Not significant. $*P < .05$ and $***P < .001$, 1-way ANOVA with the Bonferroni *post hoc* test.

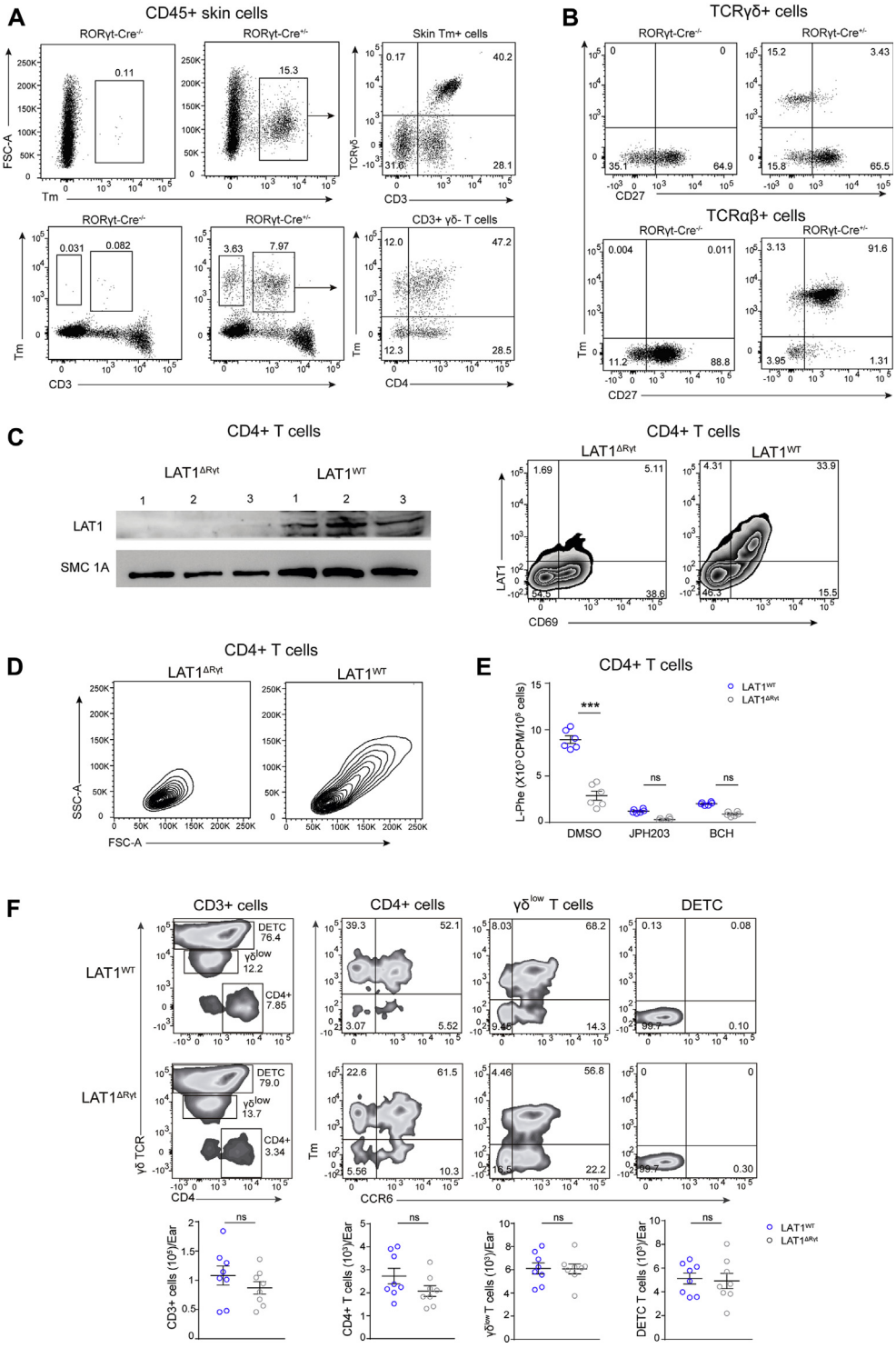

FIG E2. Characterization of immune cells with deletion of LAT1 under the control of RORγt expression. **A,** Skin Tm^+ cells of RORγt-$Cre^{-/-}$ and RORγt-$Cre^{+/-}$ mice were identified as $CD3^{low}$ γδTCR^{low} cells, CD4 T cells, and $CD3^-$RORγt^+ innate lymphoid cells. **B,** Tm expression observed in $CD27^-$ γδ T cells *(upper)* and $CD27^+$ αβ T cells *(bottom)* from RORγt-$Cre^{+/-}$ mice. **C,** LAT1 expression was assessed by using Western blotting *(left)* and FC *(right)* in activated CD4 T cells from $LAT1^{WT}$ and $LAT1^{\Delta Ryt}$ mice. **D,** Size and complexity of cells obtained as in Fig E2, *C*, were evaluated by means of FC. **E,** L-phenylalanine uptake was assessed in activated CD4 T cells from $LAT1^{WT}$ and $LAT1^{\Delta Ryt}$ mice. **F,** Expression of Tm and CCR6 in skin CD4 T cells, dermal γδ T cells ($γδ^{low}$), and epidermal γδ T cells *(DETC)* in $LAT1^{WT}$ and $LAT1^{\Delta Ryt}$ mice are shown. Absolute numbers are shown *(bottom)*. A representative experiment of 2 is shown (n = 3-4 [Fig E2, *A-D*] or n = 6 [Fig E2, *E*]). A pool of 2 independent experiments is shown (n = 4 [Fig E2, *F*]). Data are shown as means ± SEMs. *ns*, Not significant. ***$P < .001$, 2-way ANOVA with the Bonferroni *post hoc* test (Fig E2, *E* and *F*).

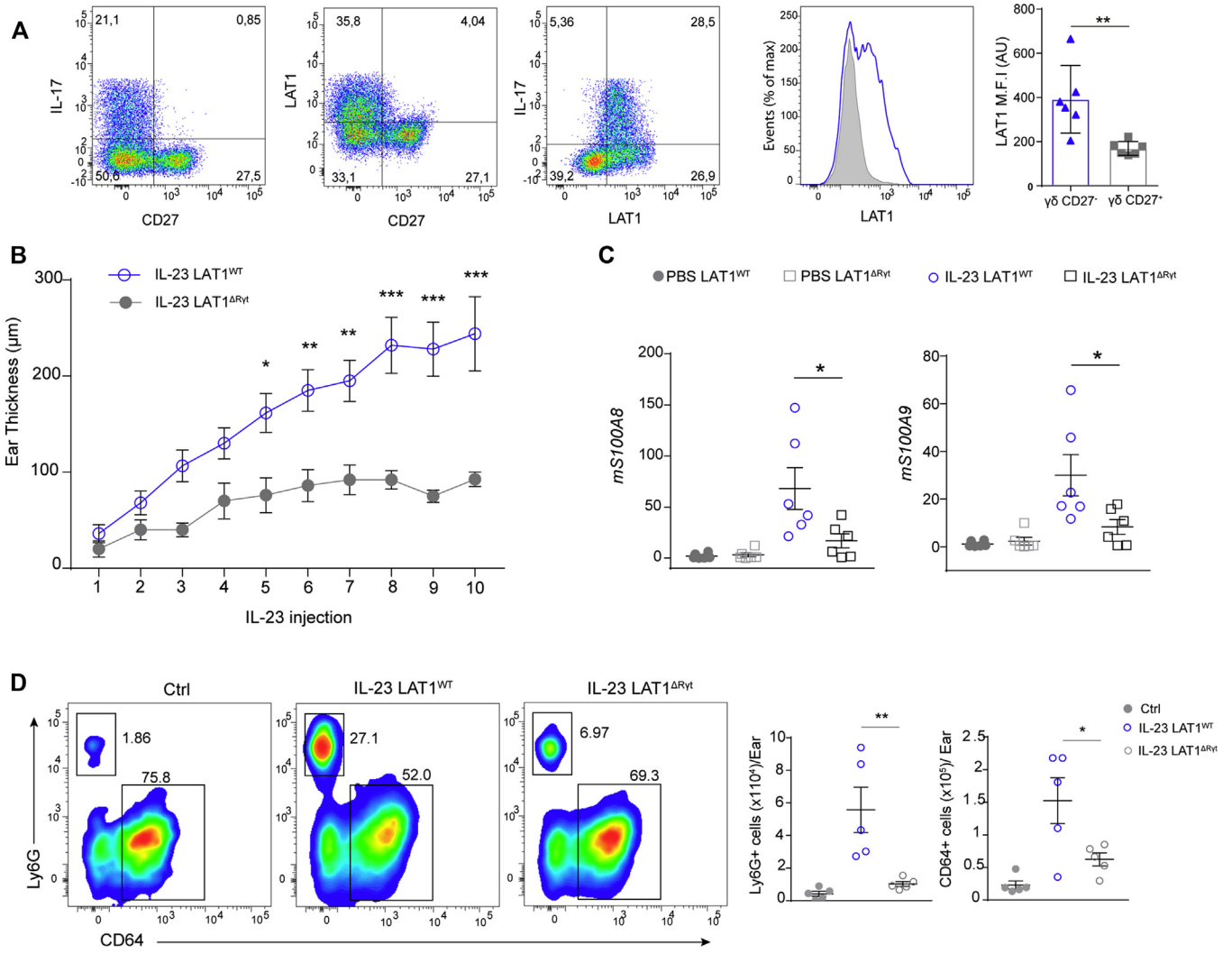

FIG E3. LAT1 expression in IL-17$^+$ γδ T cells controls the IL-23–induced psoriasis model. **A,** Dot plots of lymph node γδ T cells from WT mice after IMQ. Expression of LAT1 in CD27$^-$IL-17$^+$ cells is shown *(dot plots)* and compared with CD27$^+$ γδ T cells (*histograms* and *bars*). **B,** Ear thickness of LAT1$^{\Delta Ryt}$ and LAT1^{WT} mice assessed after IL-23 intradermal injections. **C,** Transcriptional levels of indicated genes in the skin of mice treated with PBS or IL-23. **D,** Representative density plots and density values of neutrophils (Ly6G$^+$) and macrophages (CD64$^+$) infiltrating the skin of mice treated or not with IL-23. A representative experiment of 2 individual replicates is shown (n = 5-6). Data are shown as means ± SEMs. *ns*, Not significant. **P* < .05 and ***P* < .01, 2-tailed unpaired Student *t* test (Fig E3, *A* and *B*) and 1-way ANOVA with the Bonferroni *post hoc* test (Fig E3, *C* and *D*).

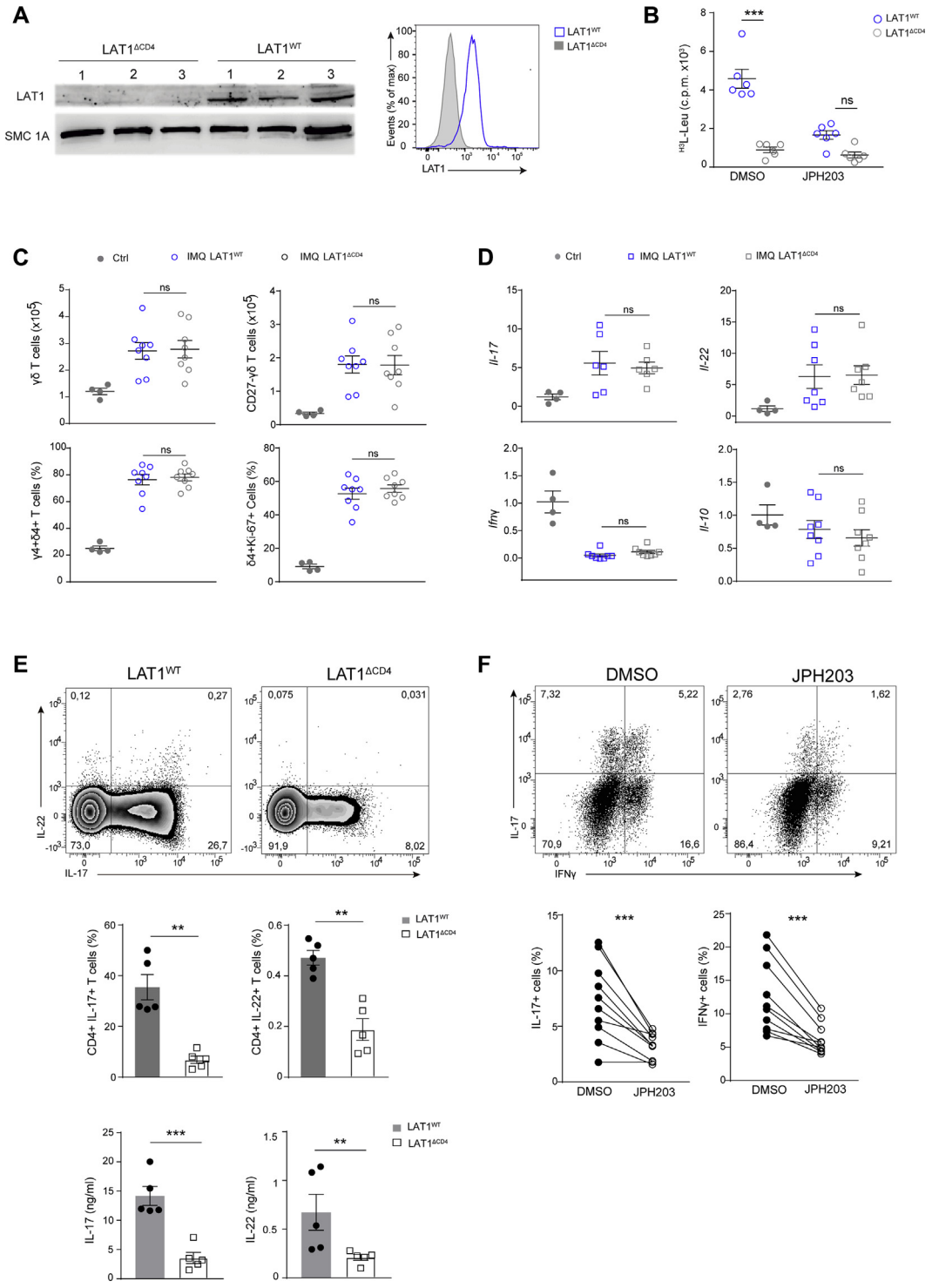

FIG E4. Deletion of LAT1 in CD4 T cells prevents T_H17 differentiation. **A,** LAT1 expression detected by means of Western blotting *(left)* and FC *(right)* in activated CD4 T cells. **B** L-Leu uptake assessed in activated CD4 T cells. **C,** Absolute numbers of γδ *(upper left)* and $CD27^-$ γδ *(upper right)* T cells and frequency of $V\gamma4^+\delta4^+$ T cells *(bottom left)* and $V\delta4^+Ki\text{-}67^+$ cells *(bottom right)* in lymph nodes. **D,** Transcriptional levels of indicated genes in γδ T cells from lymph nodes. **E,** Density plots of mouse T_H17 cells after PMA/ionomycin stimulation *(upper)*. Frequencies of IL-17– and IL-22–secreting cells *(middle)* and cytokine levels (ELISA; *bottom*) are shown. **F,** Human T_H17 cells were obtained in the presence of JPH203 or its vehicle. Representative dot plots after PMA/ionomycin stimulation *(upper)* and frequencies of IL-17– and IFN-γ–secreting cells *(bottom)* are shown. A representative experiment of 2 is shown (n = 3 [Fig E4, *A*]; n = 5-6 [Fig E4, *B* and *E*]; n = 10 [Fig E4, *F*]). A pool of 2 independent experiments is shown (n = 4 [Fig E4, *C* and *D*]). Data are shown as means ± SEMs. *ns*, Not significant. $*P < .05$, $**P < .01$, and $***P < .001$, 2-way ANOVA with the Bonferroni *post hoc* test (Fig E4, *B*), 1-way ANOVA with the Bonferroni *post hoc* test (Fig E4, *C* and *D*), and 2-tailed unpaired (Fig E4, *E*) and paired (Fig E4, *F*) Student *t* tests.

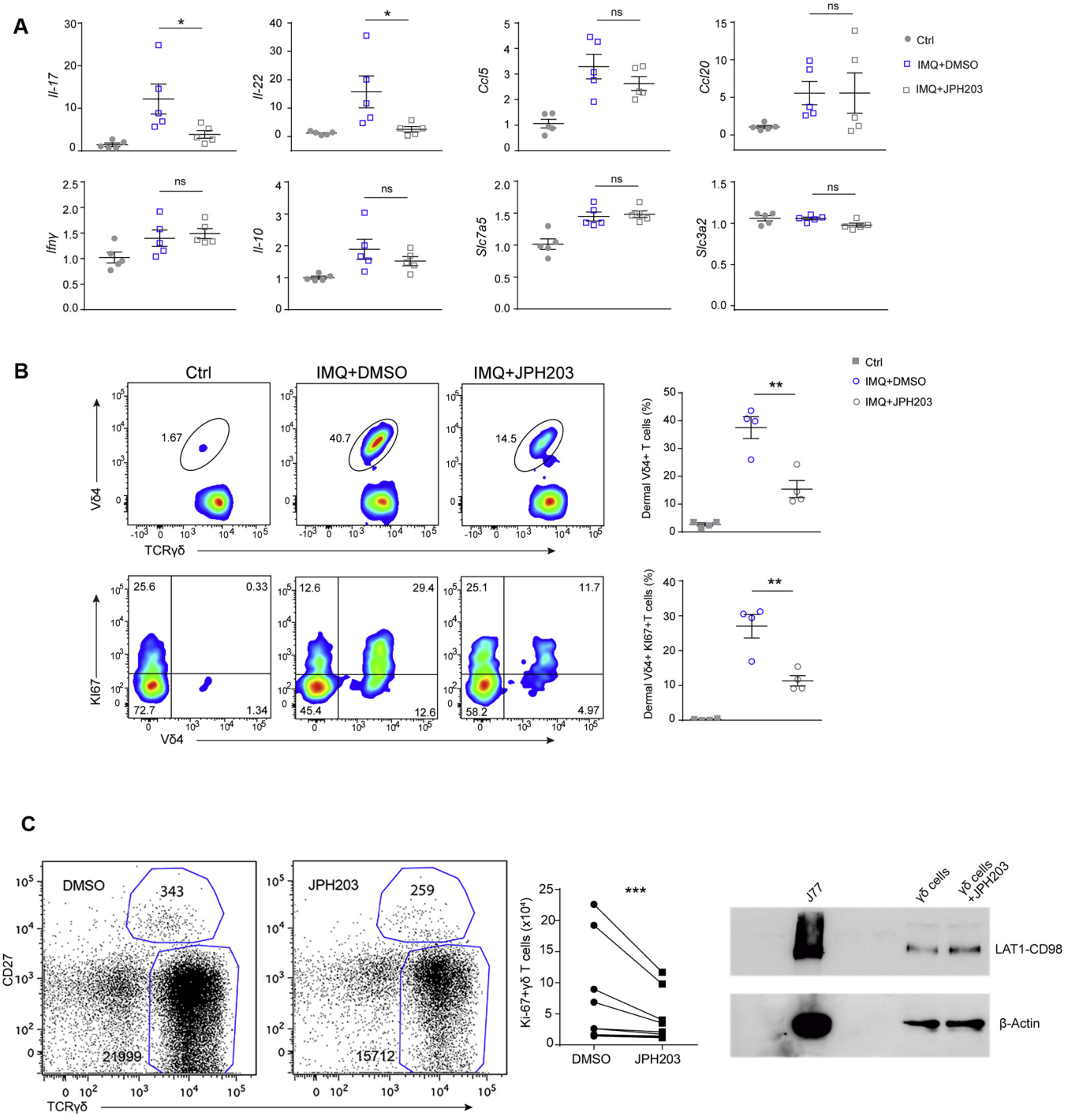

FIG E5. Effects of the LAT1 inhibitor JPH203 in immune cells. **A,** Transcriptional levels of the indicated genes induced in skin-draining lymph node after IMQ. **B,** Density plots *(left)* of the frequency of $V\delta4^+$ *(upper)* and $Ki\text{-}67^+V\delta4^+$ T cells *(bottom)* from the dermal γδ T-cell population. Frequency values are shown at *right*. **C,** Dot plots *(left)* of live human γδ T cells expanded *in vitro* with zoledronate. Values in dot plots indicate total numbers of cells detected in culture after incubation with DMSO or JPH203 normalized by the number of beads. The effect of JPH203 in total γδ T cells obtained from each patient (n = 8 patients) is shown *(middle)*. Representative western blot of purified γδ T cells from one patient to analyze the LAT1-CD98 amino acid complex is shown *(right)*. A representative experiment of 2 is shown (n = 4-5 per group). Data are shown as means ± SEMs. *ns*, Not significant. $*P < .05$, $**P < .01$, and $***P < .001$, 1-way ANOVA with the Bonferroni *post hoc* test (Fig E5, *A* and *B*) and the 2-tailed paired Student *t* test (Fig E5, *C*).

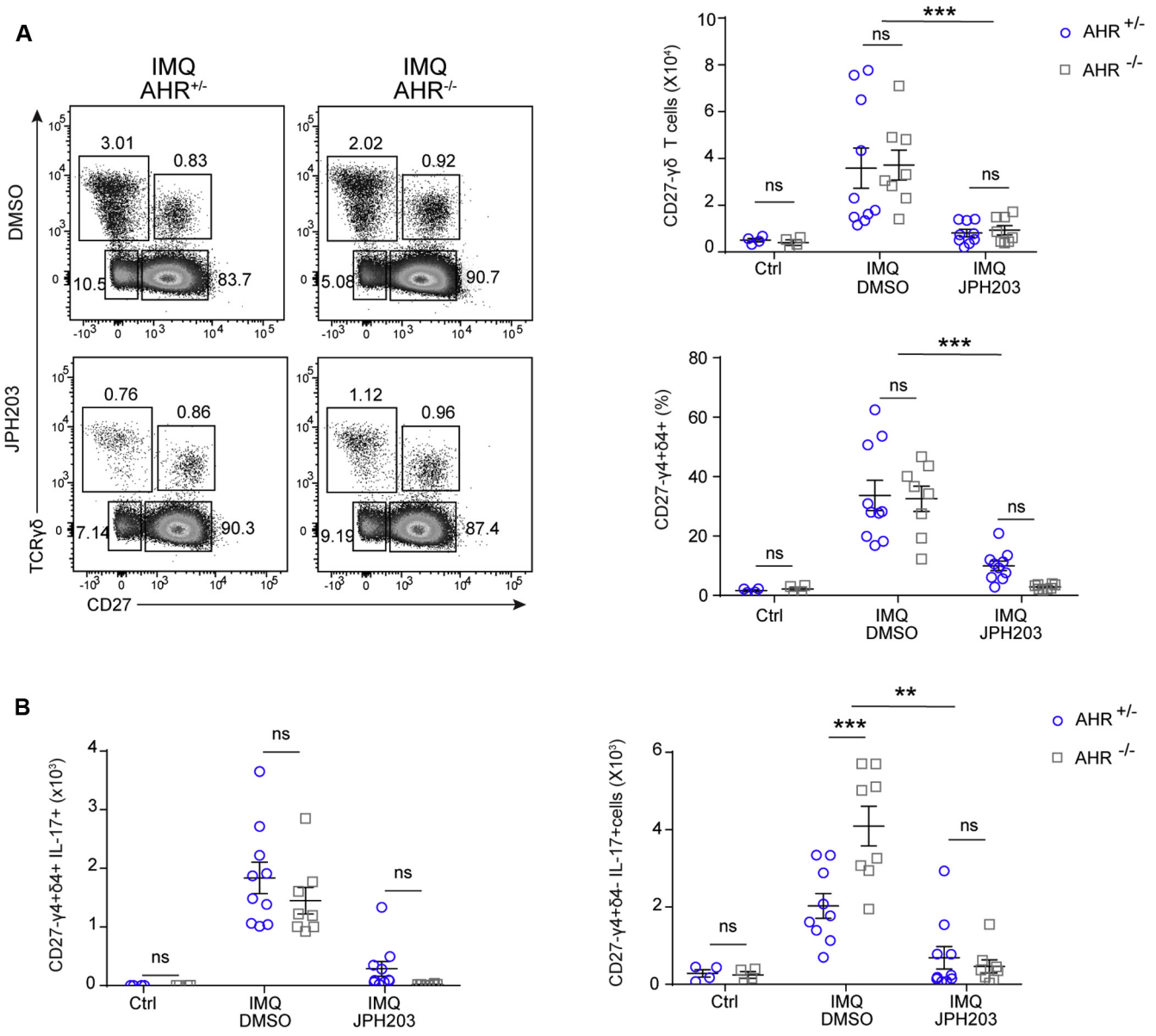

FIG E6. LAT1 inhibition controls γδ T-cell proliferation and IL-17 secretion independently of AHR expression. $AHR^{+/-}$ and $AHR^{-/-}$ mice received IMQ in the ear and were injected with Brefeldin A before death. **A,** Representative dot plots *(left)* and total numbers (*right* and *upper*) of $CD27^{-}$ γδ T cells detected in lymph nodes. Frequencies of $CD27^{-}$ $V\gamma4^{+}\delta4^{+}$ T cells (gated on $CD27^{-}$ γδ T cells) in lymph nodes are also shown (*right* and *bottom*). **B,** Total numbers of $CD27^{-}V\gamma4^{+}\delta4^{+}$ *(left)* and $CD27^{-}V\gamma4^{+}\delta4^{-}$ *(right)* T cells producing IL-17 are shown. Data pools of 2 independent experiments (n = 4) are represented. Data are shown as means ± SEMs. *ns*, Not significant. $^{**}P < .01$ and $^{***}P < .001$, 1-way ANOVA with the Bonferroni *post hoc* test.

TABLE E1. List of used antibodies

Specificity	Reactivity	Clone	Origin	Dilution	Catalog no.
CCR6	Mouse	29-2L17	BioLegend, San Diego, Calif	1:200	129818
CD3e	Mouse	145-2C11	BD Biosciences, San Jose, Calif	1:200	553066
CD4	Mouse	RM4-5	BD Biosciences	1:200	20-0042
CD8	Mouse	53-6.7	BioLegend	1:200	100766
CD11b	Mouse	M1/70	BD Biosciences	1:200	553310
CD11c	Mouse	HL3	BD Biosciences	1:200	557401
CD27	Mouse	LG.7F9	eBioscience, San Diego, Calif	1:200	25-0271-82
CD45.2	Mouse	104	BD Biosciences	1:200	560696
CD64	Mouse	X54-5/7.1	BD Biosciences	1:200	558539
CD98	Mouse	RL388	BioLegend	1:200	128210
CD98	Human, mouse	H300	Santa Cruz Biotechnology, Dallas, Tex	1:500	sc-9160
LAT1	Mouse	H-75	Santa Cruz Biotechnology	1:50	sc-134994
αCD16/αCD32	Mouse	2.4G2	Tonbo Biosciences, San Diego, Calif	1:200	70-0161
IL-17A	Mouse	TC11-18H10	BD Biosciences	1:100	559502
IL-22	Mouse	IL22JOP	eBioscience	1:100	17-7222-82
Ki-67	Mouse	B56	BD Biosciences	1:100	558615
Ly6C	Mouse	AL-21	BD Biosciences	1:200	560525
Ly6G	Mouse	1A8	BD Biosciences	1:200	551461
pS6 (Ser235/236)	Mouse	D57.2.2E	Cell Signaling, Danvers Mass	1:100	8520S
TCRαβ	Mouse	H57-597	BD Biosciences	1:200	109220
TCRγδ	Mouse	GL3	BioLegend	1:200	118118
TCR-Vδ4	Mouse	GL2	BioLegend	1:200	134905
TCR-Vγ4	Mouse	UC3-10A6	BioLegend	1:200	137704
SLC7A5	Human	pAb	Sigma-Aldrich, St Louis, Mo	1:200	HPA052673
SLC7A8	Human, mouse	pAb	Sigma-Aldrich	1:200	HPA051950
SLC43A1	Human	pAb	Sigma-Aldrich	1:200	HPA01882

TABLE E2. Sequences of primers

Gene	Specie	Forward primer	Reverse primer
Actb	Mouse	CAGAAGGAGATTACTGCTCTGGCT	TACTCCTGCTTGCTGATCCACATC
Ccl20	Mouse	ACTGTTGCCTCTCGTACATACA	GAGGAGGTTCACAGCCCTTTT
Ccr6	Mouse	ATGCGGTCAACTTTAACTGTGG	CCCGGAAAGATTTGGTTGCCT
Gapdh	Mouse	AGCTTGTCATCAACGGGAAG	TTTGATGTTAGTGGGGTCTCG
Hprt	Mouse	GCAGTACAGCCCCAAAATGG	GGTCCTTTTCACCAGCAAGCT
Ifng	Mouse	ATGAACGCTACACACTGCATC	CCATCCTTTTGCCAGTTCCT
Il10	Mouse	GCTCTTACTGACTGGCATGAC	CGCAGCTCTAGGAGCATGTG
Il17a	Mouse	TTTAACTCCCTTGGCGCAAAA	CTTTCCCTCCGCATTGACAC
Il22	Mouse	ATGAGTTTTTCCCTTATGGGGAC	GCTGGAAGTTGGACACCTCAA
Il23	Mouse	ATGCTGGATTGCAGAGCAGTA	ACGGGGCACATTATTTTTAGTCT
S100a8	Mouse	AAATCACCATGCCCTCTACAAG	CCCACTTTTATCACCATCGCAA
S100a9	Mouse	ATACTCTAGGAAGGAAGGACACC	TCCATGATGTCATTTATGAGGGC
Slc3a2	Mouse	GACACCGAAGTGGACATGAAA	GCTCCTCCTTGGATAAGCCG
Slc7a5	Mouse	CTGGATCGAGCTGCTCATC	GTTCACAGCTGTGAGGAGC

Review article

Dissecting the complexity of γδ T-cell subsets in skin homeostasis, inflammation, and malignancy

Raquel Castillo-González, MS,[a]* Danay Cibrian, PhD,[a,b,c]* and Francisco Sánchez-Madrid, PhD[a,b,c] *Madrid, Spain*

γδ T cells are much less common than αβ T cells, accounting for 0.5% to 5% of all T lymphocytes in the peripheral blood and lymphoid tissues in mice and humans. However, they are the most abundant T-lymphocyte subset in some epithelial barriers such as mouse skin. γδ T cells are considered innate lymphocytes because of their non-MHC restricted antigen recognition, as well as because of their rapid response to cytokines, invading pathogens, and malignant cells. Exacerbated expansion and activation of γδ T cells in the skin is a common feature of acute and chronic skin inflammation such as psoriasis and contact or atopic dermatitis. Different γδ T-cell subsets showing differential developmental and functional features are found in mouse and human skin. This review discusses the state of the art of research and future perspectives about the role of the different subsets of γδ T-cells detected in the skin in steady-state, psoriasis, dermatitis, infection, and malignant skin diseases. Also, we highlight the differences between human and mouse γδ T cells in skin homeostasis and inflammation, as understanding the differential role of each subtype of skin γδ T cells will improve the discovery of new therapies. (J Allergy Clin Immunol 2020;■■■:■■■-■■■.)

Key words: *γδ T cells, skin, psoriasis, atopic dermatitis, infection, cancer*

Abbreviations used

ACD:	Allergic contact dermatitis
AD:	Atopic dermatitis
BCG:	Bacillus Calmette-Guérin
CCR:	Chemokine receptor
CD:	Contact dermatitis
CHS:	Contact hypersensitivity
DETC:	Dendritic epidermal T cell
FGF:	Fibroblast growth factor
γδ17 T cell:	IL-17–secreting γδ T cell
γδIFN-γ T cell:	IFN-γ–secreting γδ T cell
HSV-1:	Herpes simplex virus-1
ICD:	Irritant contact dermatitis
IGF-1:	Insulin-like growth factor 1
IL-23R:	IL-23 receptor
IPP:	Isopentenyl pyrophosphate
KGF:	Keratinocyte growth factor
mTOR:	Mammalian target of rapamycin
PD-1:	Programmed death-1
RhoH:	Ras homolog gene family H
RORγt:	Retinoic acid–related orphan receptor γt
sdc1:	Syndecan-1
TCR:	T-cell receptor
TLR:	Toll-like receptor

The skin is the largest barrier in the organism that protects against environmental factors and external pathogens. Both αβ and γδ T cells exert crucial functions to maintain skin homeostasis, but they are also important mediators in autoimmune skin disorders.[1] γδ T cells are *bona fide* gatekeepers of skin homeostasis and wound healing,[2] with essential roles in psoriasis,[3] dermatitis,[4] skin infections,[5] and cancer diseases.[6] In recent years, several studies have described the origin and function of γδ T-cell subsets in homeostasis and skin-related diseases, which will be reviewed later in this article.

ORIGIN, MARKERS, AND FUNCTIONAL DIFFERENTIATION OF MOUSE AND HUMAN γδ T CELLS

γδ T cells arise from the same progenitor as αβ T cells; however, unlike αβ T cells (which exit the thymus as naive T cells), fetal and newborn γδ T cells acquire their effector function in the thymus. T-cell receptor (TCR) signal strength is a crucial determinant of their functional lineage identity (reviewed in Munoz-Rui et al).[7] In mice, most γδ T lymphocytes express CD27 and secrete IFN-γ (γδIFN-γ T cells), whereas IL-17 production is restricted to $CD27^-$ γδ T cells (γδ17 T cells).[8,9] Mouse $CD27^+$ IFN-γ–secreting γδ T cells express the specific markers T-bet and NK1.1, whereas $CD27^-$ γδ17 T cells express the transcription factor retinoic acid–related orphan receptor γt (RORγt) and the chemokine receptor 6 (CCR6).[10] Moreover, mouse γδ T cells are divided into several subsets depending on their

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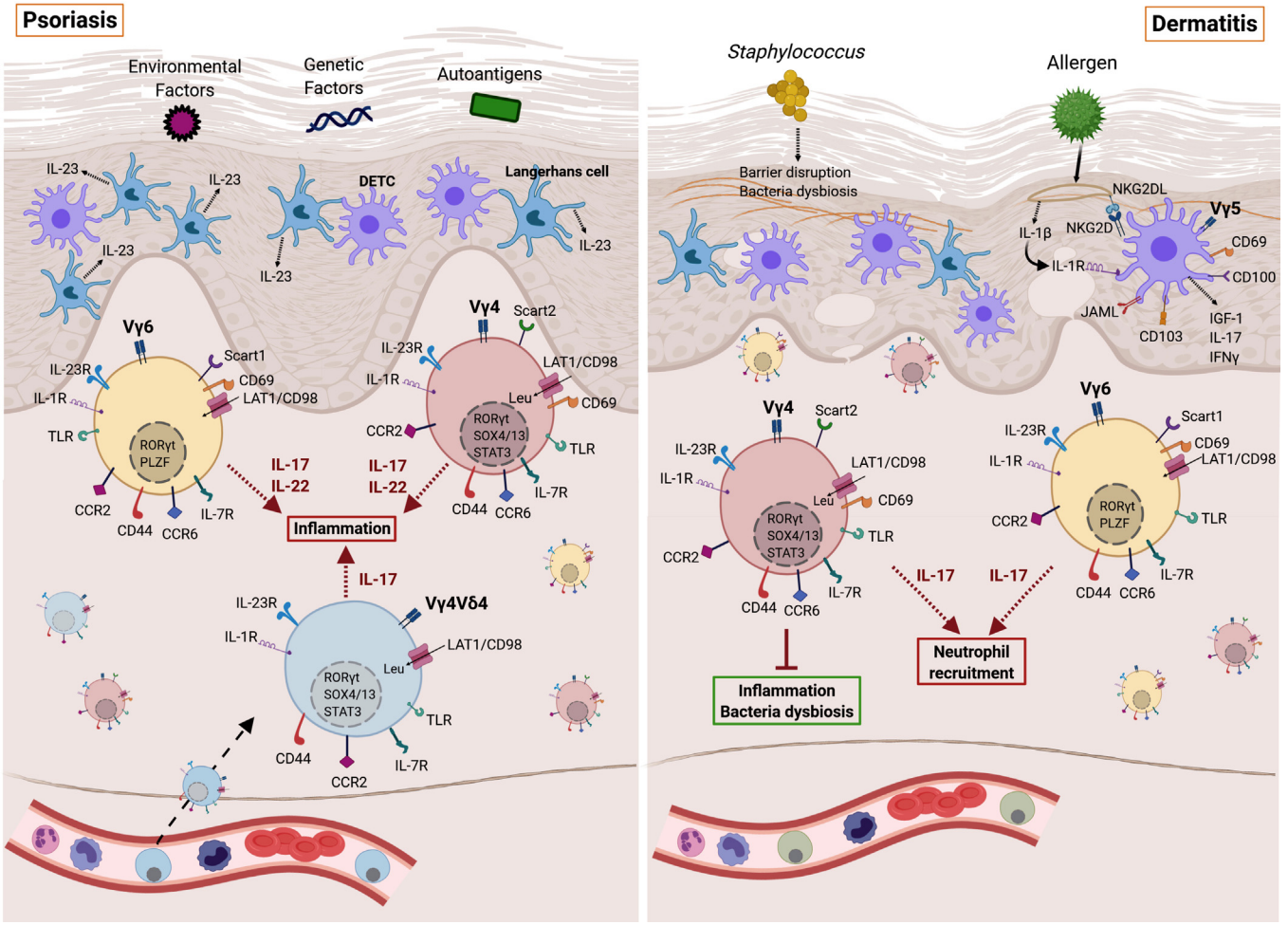

FIG 1. Mouse skin γδ T-cell subsets in psoriasis and atopic dermatitis. Dermal γδ T cells are central players in different inflammatory skin diseases, such as psoriasis and allergic/atopic dermatitis. DETC subsets do not actively participate in the development of psoriasis (*left panel*). Both experimental models, namely, imiquimod- and IL-23–induced psoriasis, involve the activation of natural dermal Vγ6 and Vγ4 T cells by IL-23 and IL-1β, thus inducing IL-17 and IL-22 secretion. Expansion of an inducible Vγ4Vδ4 T-cell population in the lymph nodes and its migration to the skin are characteristic of the imiquimod (IMQ) model of psoriasis. In allergic dermatitis (*right panel*), IL-1β, NKG2D, and TCR signaling are required for DETC activation and release of proinflammatory mediators. Dermal Vγ4 T cells participate in the control of bacterial dysbiosis associated with barrier dysfunction, which leads to AD. The role of the Vγ6 subset in AD has not been clarified. In addition, the secretion of IL-17 by skin- resident γδ T cells is pathologic in AD and CHS models. This figure shows intracellular and extracellular markers that are expressed in the different skin γδ T-cell subsets. (This figure was created with BioRender software [available at BioRender.com].)

expression of Vγ chains, which is associated with their tissue tropism and effector function[6] (Fig 1 and Table I). Herein, we have used the Tonegawa nomenclature to catalog mouse Vγ chains.[11] Mouse $CD27^{+}$ γδIFN-γ T cells can express Vγ1 or Vγ4, whereas $CD27^{-}$ γδ17 T cells express Vγ4 or Vγ6.[6]

Different subtypes of γδ T cells are found in mouse skin in steady state, including dendritic epidermal T cells (DETCs)[12] and dermal-resident Vγ6 and Vγ4 T-cell populations, which secrete IL-17 and are in a proportion that is close to 1:1.[3,13,14] DETCs exclusively bear Vγ5Vδ1 TCR chains and account for 90% of lymphocytes in mouse epidermis.[12] DETCs are sessile cells with a dendritic form that remain in close contact with surrounding keratinocytes and Langerhans cells.[2,15] This population originates exclusively from the yolk sac at embryonic day E13.5 and persists throughout the life of the mouse by limited expansion in the epidermis.[16] DETCs are radioresistant cells that recognize skin-derived self-antigens and participate in immune surveillance.[17] Activated DETCs can release several cytokines, such as IL-13, IFN-γ, TNF-α, IL-2, and IL-17, under specific pathogenic situations.[18-20] At steady state, DETC turnover is slow, but following skin injury, clonal proliferation of tissue-resident cells supports their replenishment and homeostasis.[16] Importantly, a population equivalent to DETCs has not been found in human epidermis yet, and their frequency is variable in different mouse strains.[21,22]

Dermal Vγ6 and Vγ4 T-cell populations predominantly express IL-17A and IL-22.[3,14,23] They share a very similar program of gene expression in steady state that includes phenotypic markers such as RORγt, CCR6, CD44, and CD69.[24] However, they differ in terms of their origin, as well as in terms of their functional requirements.[25] Vγ6 T cells are generated solely in the thymic second wave around embryonic day E16, and they

exclusively express the Vδ1 TCR chain. On the other hand, Vγ4 T cells appear in the late fetal stage and newborn thymus, and they have shown variable Vδ partners. Importantly, the expression of Scart1 can be also used to distinguish IL-17$^+$ Vγ6 T cells, whereas the expression of Scart2 can identify IL-17$^+$ Vγ4 T cells.[24] Moreover, dermal Vγ4 T cells showed higher expression of CD9, galectin 3, and IL-17F, whereas expression of programmed cell death 1 (PD-1) receptor and CCR2 is more abundant in dermal Vγ6 T cells.[24] Importantly, IL−17 secretion can be induced by IL-23 stimulation in Vγ6 and Vγ4 T cells, but Vγ4 T cells are the main producers of IL-17 after costimulation with IL-1β.[25] During the past few years, experiments using chimeric and parabiotic mice have indicated that both dermal Vγ4 and Vγ6 T subsets are radioresistant, cannot be reconstituted with bone marrow cells, and indeed require fetal thymocytes for their reconstitution.[13,14] Whereas dermal Vγ6 T cells are considered *bona fide* tissue-resident cells that do not recirculate out of the skin,[24] the dermal Vγ4 T subset can migrate at a low rate to lymph nodes in steady state.[23,26] The migration of Vγ4 T cells is enhanced during skin infection or during contact hypersensitivity reaction.[23,26] Migration of the dermal Vγ4 subset to skin-draining lymph nodes is also induced by CFA during induction of an experimental autoimmune encephalomyelitis model, in which Vγ4 cells are the main source of IL-17.[27] The application of the Toll-like receptor (TLR) 7/9 (TLR7/9) agonist imiquimod in the skin expands the population of Vγ4Vδ4 T cells in the lymph nodes that migrate to the skin in a fingolimod-sensitive manner.[28-30] The imiquimod-induced expanded population of Vγ4Vδ4 T cells is of extrathymic origin and is derived from bone marrow precursors.[29] The numbers of these extrathymic/bone marrow or "inducible" Vγ4Vδ4 T cells are also expanded in experimental autoimmune encephalomyelitis models after intradermal injection of CFA.[31] Importantly, inducible Vγ4Vδ4 T cells showed increased IL-17 secretion as compared with fetal/newborn or "natural" Vγ4 and Vγ6 T cells, required CCR2 but not CCR6 to traffic to the skin, and established a long-lived skin memory response that exacerbated the secondary inflammation.[29] Inducible Vγ4 T cells are reprogrammed from the CD27$^+$IFN-γ–biased counterpart in the periphery owing to the action of IL-23 and IL-1β.[31,32] Interestingly, the differentiation of natural versus inducible γδ17 T cells requires distinct cytokines. Thymic γδ17 T-cell development does not require IL-23, whereas TGF-β1 and IL-7 are essential,[33,34] and it is inhibited by IL-15.[35] On the other hand, IL-23 is essential for expansion of the number of bone marrow–inducible γδ17 T cells after IL-1β stimulation, but TGFβ and IL-6 are not required.[31,32] Further studies are required to evaluate whether a similar reprogramming of IFN-γ–biased γδ T cells can mediate expansion or maintenance of the numbers of inducible γδ17 T cells in other diseases.

In recent years, efforts have been made to identify genetic and environmental factors regulating the generation and maintenance of different mouse skin γδ T cells. Generation of Vγ5Vδ1 epidermal γδ T cells requires the engagement of Skint-1 thymic epithelial cells. This mechanism of positive selection promotes differentiation of IFN-γ–producing γδ T cells and suppresses SOX13 and RORγt expression, thus preventing the development of γδ17 T cells.[19] Mice deficient in nuclear factor-κB–inducing kinase also showed an altered development of DETCs.[36] Besides, DETC development requires IL-7 and IL-15 cytokines,[13,37,38] as well as expression of the transcription factor aryl hydrocarbon receptor for their maintenance.[39,40] Several transcription factors can regulate the development and maintenance of γδ17 T cells; they include SOX4 and SOX13 (Vγ4 T cells),[4,41] PLZF (Vγ6 T cells),[42] and Notch, Heb, and cMAF (both subsets).[43-45] The spontaneous mutation of SOX13 was detected in the mouse lines B6.SJL/NCI and SJL/Tac, causing the lack of Vγ4 T-cell population.[4] These mouse lines that express the CD45.1 haplotype in a C57BL6 background are frequently used for the generation of chimeric mice. However, there are B6.SJL strains available (B6.SJL/Jax and B6.SJL/CR) without this phenotype.[4] Thus, it is necessary to consider the origin of the B6.SJL strain of mice used for better experiment planning and interpretation of results regarding the role of different populations of γδ17 T cells in homeostasis and pathologic situations.

On the other hand, human γδ T cells can be divided into 4 main populations based on TCRδ chain expression (δ1, δ2, δ3, and δ5) (Table II). Vδ2 T cells coexpressing the Vγ9 chain constitute the most frequent subset of the γδ T-cell population in the peripheral blood and secondary lymphoid organs of fetal and adult individuals. Vγ9Vδ2 T cells are developed in the fetal liver and thymus.[46-48] Although Vγ9Vδ2 T cells localize primarily in the circulation, they can be recruited to inflamed tissues where they participate in pathogen and tumor clearance; however, they can also promote inflammation (Fig 2). Vγ9Vδ2 T cells recognize nonpeptidic pyrophosphate compounds (phosphoantigens), including isopentenyl pyrophosphate (IPP) and microbial-derived compounds such as (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate.[49] Cancer cells have shown upregulation of IPP levels that can be further increased via amino bisphosphonate drugs blocking IPP catabolism, such as zoledronate.[50] Activation of Vγ9Vδ2 T cells by phosphoantigens requires the direct binding of butyrophilin (BTN)2A1 with the Vγ9 TCR chain.[51,52] Expression of BTN3A1, which senses intracellular phosphoantigens and is directly associated with BTN2A1 in the activation-membrane complex, is also required for optimal response of Vγ9Vδ2 T cells.[53-55] Both fetal and adult Vγ9Vδ2 T cells readily expand and produce IFN-γ and cytotoxic molecules in response to stimulation with (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate.[46]

Human Vδ2$^-$ γδ T cells are mainly represented by Vδ1 T cells, and to a lesser extent, by Vδ3 and Vδ5 T cells. The Vδ1 T-cell subset is heterogeneous in the use of Vγ chains and preferentially resides in epithelial tissues such as the skin and intestine.[56] Representative antigens recognized by Vδ1 T cells include MHC class I–related molecule A/B and UL16-binding protein molecules, which are expressed on stressed normal cells and tumor cells.[57] Besides, a minor subset of human Vδ1 T cells can recognize the MHC-related class Ib molecules CD1c and CD1d, which are involved in lipid presentation.[58] Moreover, Vδ1 T cells can also be activated by engagement of natural cytotoxicity receptors such as NKp30, NKp44, and NKp46.[59] Recognition of the MHC class I–related molecule A/B by Vδ1 T cells is mediated by the NKG2D receptor and by the TCR.[60] Importantly, clonal expansion of Vδ1 cells is detected in response to cytomegalovirus infection, indicating a possible TCR-dependent selection mediated by microbial encounters throughout life.[61] The recognition of stress antigens by Vδ1 T cells indicates that noninfectious events such as cancer might also shape their repertoire.[62] Finally, human Vδ3 T cells are abundant in the liver[62] and are activated by CD1d-mediated recognition of glycolipids.[63]

In humans, a natural IL-17–biased γδ T-cell population, such as the dermal Vγ4 and Vγ6 T cells found in mice, has not yet been

TABLE I. Mouse skin γδ T-cell subsets in homeostasis and inflammation

Skin γδ T-cell subset	Vδ pair	Origin	Characteristic	Skin localization	Effector molecule
DETC (Vγ5)	Vδ1	First thymic wave (E d. 13.5)	$CD27^-$, $CD69^+$, T-bet^+, $NKG2D^+$, $JAML^+$, $CD100^+$, $CD103^+$ Dendritic form Tissue-resident Radioresistant Require the engagement of Skint-1^+ thymic epithelial cells for their generation Require IL-7, IL-15, and AHR expression for development and maintenance	Epidermis	IGF-1 KGF-1 KGF-2 IFN-γ TNF-α IL-2 IL-13 IL-17
Fetal-derived Vγ6	Vδ1	Second thymic wave (E d. 16)	$CD27^-$, IL-$23R^+$, $ROR\gamma t^+$, $CCR6^+$, $CD69^+$, $CD44^+$, Scart1+, $cMAF^+$, $PLZF^+$ Tissue-resident Radioresistant High expression of PD-1 Require IL-7, TGF-β for development	Dermis	IL-17 IL-22 FGF-9
Fetal-derived Vγ4	Variable	Late fetal and newborn thymus	$CD27^-$, IL-$23R^+$, $ROR\gamma t^+$, $CCR6^+$, $CD69^+$, $CD44^+$, $Scart2^+$, $SOX13^+$, $SOX4^+$, $cMAF^+$ Low ratio of migration to lymph nodes Radioresistant High expression of CD9, galectin-3, and IL-17F Require IL-7, TGF-β for development	Dermis	IL-17 IL-22
Bone marrow–derived Vγ4	Vδ4	Bone marrow	Skin homing is dependent on CCR2 expression $CD27^-$, $ROR\gamma t^+$ expression Stablish long-lived memory-like response in the skin Require IL-23 and IL-1β for their reprogramming from $CD27^+$ γδ T cells	Dermis	IL-17

E d., Embryonic day.

TABLE II. Human skin γδ T-cell subsets in homeostasis and inflammation

Skin γδ T-cell subsets	Vγ pair	Origin	Characteristic	Skin localization	Effector molecule
Vδ1	Variable	Fetal thymus	$NKG2D^+$, $NKp30^+$, $NKp44^+$, $NKp46^+$ Recognize MICA, MICB, ULBPs, and glycolipids Dominant subset in healthy skin	Epidermis and dermis	IGF-1 KGFs IFN-γ TNF–α Cytotoxic molecules
Vδ2	Vγ9	Fetal liver and thymus	Recognize pAgs (IPP and HMBPP) by interaction of TCR with BTN2A1/BTN3A1 complex. Dominant subset in peripheral blood and lymphoid organs	Epidermis and dermis	IFN-γ TNF-α IL-17A Cytotoxic molecules

MICA, MHC class I-related molecule A; *MICB*, MHC class I-related molecule B; *pAg*, phosphoantigen; *ULBP*, UL16-binding protein.

identified. However, γδ17 T cells appear to be associated with various disease states.[3,64] Vγ9Vδ2 T cells can be polarized under the appropriate conditions to secrete IL-17 and IFN-γ, indicating the plasticity of this population.[65] Recently, a subset of Vδ2 T cells expressing high levels of CD26 and low levels of CD94 expression has been shown to respond to IL-23.[66]

Dysregulated function and number of γδ T cells have been associated with skin disruption of homeostasis and, therefore, with the development of different skin disorders, as is discussed in the following sections.

ROLE OF γδ T CELLS DURING HOMEOSTASIS AND WOUND HEALING RESPONSE

As the complexity of mouse skin γδ T-cell subsets has increased, their individual role in homeostasis and repair has been studied. $TCR\delta^{-/-}$ mice, which are depleted of all subsets of skin γδ T cells, showed a significant delay in the wound healing rate.[67] The dendritic morphology of DETCs allows frequent contacts with neighboring cells and continuous scanning for antigens in the skin surface.[17] Thus, DETCs can recognize antigen expressed on keratinocytes following injury and participate in tissue repair through the local production of the keratinocyte growth factors (KGFs) KGF-1 and KGF-2, which are also referred to as fibroblast growth factors FGF-7 and FGF-10, respectively.[2,67] Besides, DETCs express insulin-like growth factor 1 (IGF-1), which exerts a relevant function in the control of keratinocyte apoptosis.[68] DETC homeostasis is also regulated by a feedback loop in which IGF-1 secretion by DETCs induces mammalian target of rapamycin (mTOR)-mediated IL-15 secretion by keratinocytes, which upregulate IGF-1 release by DETCs after skin injury.[69] Additionally, DETCs can control transepidermal water loss under dry conditions.[70]

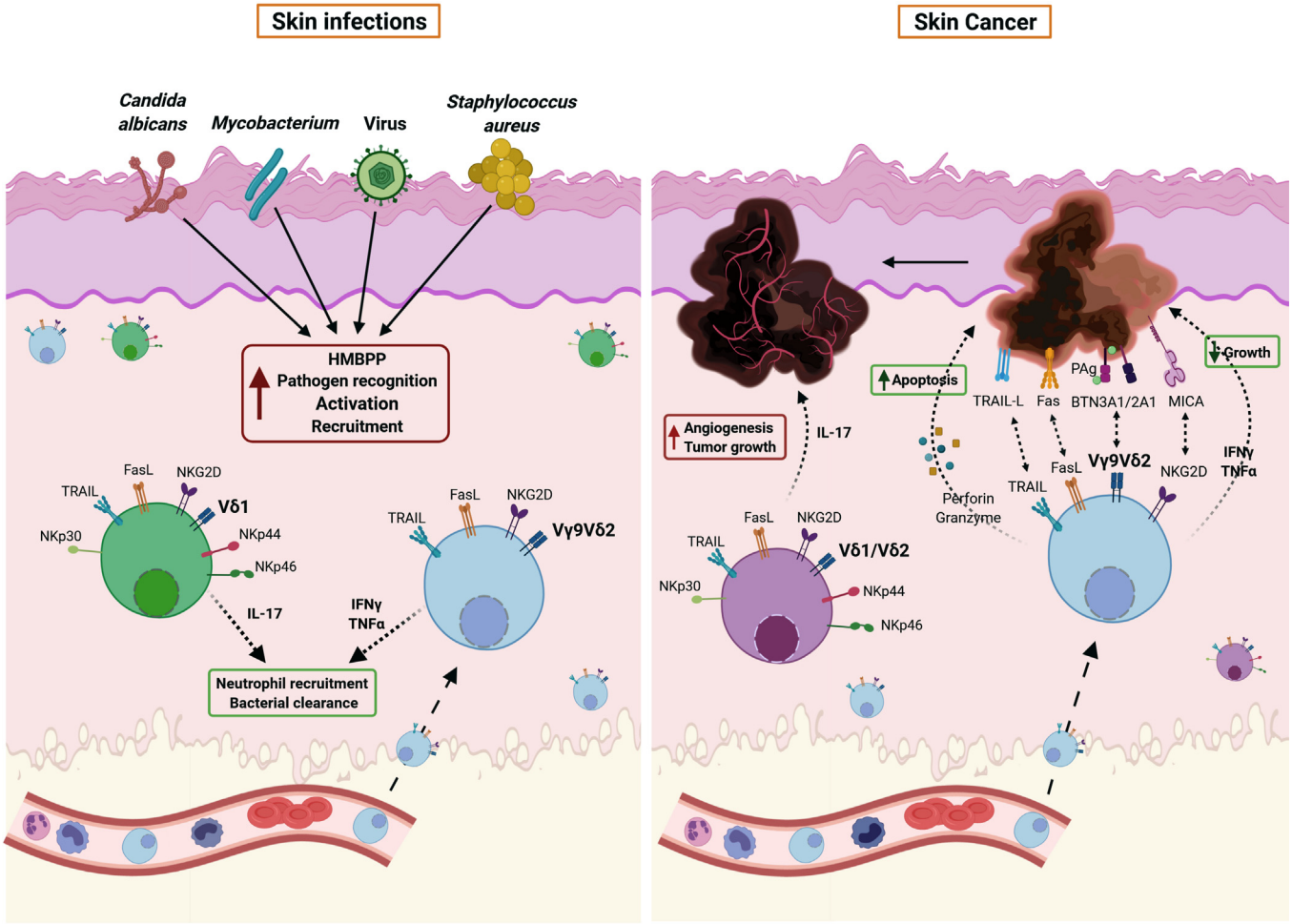

FIG 2. Role of human γδ T-cell subsets in the progression of cancer and skin infections. Circulating Vγ9Vδ2 T cells can be immediately recruited to the skin after infection with several pathogens, including virus, *Mycobacterium*, *C albicans*, and *S aureus* (*left panel*). Pathogen-activated γδ T cells promote bacterial clearance and neutrophil recruitment, thus participating in resolution of the disorder. The numbers of Vδ1 T cells are expanded in *C albicans* infection, and they secrete IL-17. In cancer (*right panel*), the direct recognition of malignant antigens by different receptors expressed in Vγ9Vδ2 T cells can induce the secretion of IFN-γ and TNF-α and can also mediate the direct lysis of tumor cells. Thus, Vγ9Vδ2 T cells are a potent tool to control tumor growth and expansion. The presence of γδ17 T cells in the tumor microenvironment contribute to tumor angiogenesis and growth, promoting the progression of cancer, and they are an important target for immunotherapy. (This figure was created with BioRender software [available at BioRender.com].) *HMBPP*, (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate.

Besides the relevance of DETCs in wound healing response, both Vγ4 and Vγ6 T cells are the predominant subsets in late-stage wounds.[71] At this time point in wound healing, Vγ6 T cells are the primary source of FGF-9, which regulates skin repair and hair follicle regeneration.[71] After injury, IL-17 secreted by Vγ4 T cells induces the production of IL-23 and IL-1β by keratinocytes, which indirectly dampens IGF-1 production by DETCs and delays wound healing.[69,72] Keratinocyte-derived mitochondrial damage–associated molecular patterns can activate dermal γδ17 T-cell populations, increasing their TLR2 and TLR4 expression, as well as the release of IL-1β, IL-6, and growth factors (PDGF and VEGF).[73] Hence, endogenous ligands that originate as a result of injury can induce activation of all population of skin γδ T cells, promoting inflammation as well as healing and remodeling events.[73]

Human γδ T cells also play key roles in maintaining skin integrity by regulating keratinocyte proliferation and homeostasis. Vδ1 cells are the dominant subtype observed in healthy skin; similar to mouse DETCs, they produce IFN-γ and KGFs.[74-76] In addition, epidermal-resident Vδ1 T cells become activated during acute tissue damage and participate in wound repair by actively producing IGF-1.[76]

ROLE OF γδ T CELLS IN IMMUNE-MEDIATED SKIN DISEASES

Psoriasis

Psoriasis is a frequent and chronic inflammatory skin disease with a global prevalence of 2% to 3%.[77] The relevance of the IL-23/IL-17 axis and γδ T cells in psoriasis has been proved in mouse models of psoriasis induced by imiquimod or IL-23 intradermal injection.[3,78] Fetal/newborn-derived Vγ4 and Vγ6 T cells, both of which secrete IL-17 and IL-22, mediate skin inflammation in

the imiquimod model.[25] Dermal Vγ6 T cells are clearly pathogenic in the model of psoriasis induced by imiquimod. However, Vγ4 T cells are considered more relevant because they cluster both fetal/newborn-derived (natural) and bone marrow–derived (inducible) populations, showing increased production of IL-17 and marked expansion in the lymph nodes (Fig 1).[4,25,30] Interestingly, in the absence of IL-12, the expansion of IL-17$^+$ Vγ4 T cells is blunted and IL-17$^+$ Vγ6 T cells are enriched and induce an exacerbated imiquimod-induced skin inflammation.[79] The role of natural Vγ6 and Vγ4 T cells in the IL-23–induced model of psoriasis has been shown.[78] However, whether a similar expansion of bone marrow–derived Vγ4 T cells is involved in the IL-23-psoriasis model has not yet been reported. The role of DETCs in a psoriasis model has been ruled out because these cells do not secrete IL-17 and IL-22 in the imiquimod or IL-23–induced murine models.[3,23]

Signaling and regulatory molecules in γδ T lymphocytes in psoriasis. Because of the critical role of γδ17 T lymphocytes in psoriasis, several studies aimed to identify different molecules that regulate their homeostasis, differentiation, and function. STAT3, which is downstream of the IL-6, IL-21, and IL-23 receptors, is important in the differentiation of T_H17 cells. However, STAT4 is primarily associated with IL-12 signaling and is required for the establishment of T_H1 cell lineage. Although, STAT3 is dispensable for the thymic development of γδ17 T cells,[44] it is necessary for the proliferation and optimal secretion of IL-17A, IL-17F, and IL-22 by Vγ4 T cells but not Vγ6 T cells in imiquimod-induced inflammation.[80,81] Hence, mice deficient in STAT3, but not in STAT4, were protected against imiquimod-induced psoriatic features.[80] However, γδ17 T cells from STAT4-deficient mice cannot produce IL-17F. Therefore, both STAT members play critical roles in the complete effector functions of γδ17 T cells.[80]

The inflammation and increase of Vγ4 T cells observed after imiquimod treatment were impaired in Ras homolog gene family H (RhoH)-deficient mice.[82] RhoH is an adaptor protein that mediates tyrosine kinase Syk recruitment to TCRγδ, which in turn activates the PI3K/Akt signaling pathway in γδ T cells.[82] The inhibition or genetic modification of the PI3K molecule prevents differentiation of γδ17 T cells[83,84] without impaired development of IFN-γ–producing γδ T cells.[82] Interestingly, Zap70, another tyrosine kinase required for the development of αβ T cells, failed to functionally substitute Syk functions in the development of γδ17 T cells.[82] All of these observations indicate that the TCR-induced Syk/PI3K/Akt pathway is essential for determination of γδ17 T-cell fate and development of imiquimod-induced psoriasis.

Furthermore, nuclear factor-κB–inducing kinase controls IL-17 secretion in the dermal pool of γδ T cells in steady state and after imiquimod treatment.[36] SOX13 deletion, which halts development of fetal and bone marrow–originated Vγ4 T cells, dampens psoriasis.[4] Dermal γδ17 T cells also express heparin sulfate proteoglycan syndecan-1 (sdc1); sdc1 controls γδ17 T-cell homeostasis, proliferation, and IL-17 production, becoming a protective factor against skin inflammation mediated by imiquimod. The absence of sdc1 increases proliferation and dampens apoptosis of γδ17 T cells.[85] Similarly, dermal γδ17 T cells constitutively express PD-1, which is upregulated following imiquimod application, whereas keratinocytes express its ligand programmed death ligand 1. Mice deficient in PD-1 display exacerbated skin inflammation due to increased levels of IL-17A and IL-22 produced by γδ T cells.[86] CD109, which is a glycosylphosphatidylinositol-anchored membrane protein that is highly expressed in healthy skin, acts as a negative regulator of the IL-23/IL-17 axis and Vγ4 and Vγ6 T cells activation by cutaneous microbiota. Hence, CD109-deficient mice develop a spontaneous skin inflammation and an exacerbated imiquimod-induced psoriasis.[87]

During the past few years, immunometabolism studies have discovered several metabolic pathways involved in immune T-cell activation and function in the skin.[88-90] The expression of LAT1, which is the main transporter of L-Leu in lymphocytes, is upregulated in γδ17 T cells following IL-23 and IL-1β coestimulation and regulates their proliferation.[28] Specific deletion of LAT1 in γδ17 T cells prevented mTOR activation and decreased IL-17 and IL-22 release, dampening imiquimod-induced psoriasis. Similarly, mTOR inhibition by systemic administration of rapamycin, decreases IL-17$^+$ Vγ4δ4 T-cell proliferation.[28] Moreover, CD69-deficient mice show an attenuated response to IL-23–induced psoriasis. This is due to the control of L-Trp uptake through LAT1-CD98, which favors aryl hydrocarbon receptor–mediated IL-22 secretion.[91]

Furthermore, psoriasis is associated with many comorbidities, such as cardiovascular diseases, obesity, and metabolic syndrome.[92] Intake of a high-fat diet increases dermal levels of IL-17$^+$ Vγ4 T cells and aggravates imiquimod-induced psoriasis.[93] Adipose tissue secretes many bioactive molecules, called adipokines (adiponectin, leptin, omentin, IL-1β, and TNF-α, among others), which exert proinflammatory and anti-inflammatory roles in the metabolic syndrome.[94] Adiponectin exerts a protective role in psoriasis induced by imiquimod and IL-23 by directly suppressing IL-17 secretion by Vγ4 T cells via AdipoR1.[95]

Some studies have also addressed the influence of circadian rhythm alterations in the development of psoriasis. The main gene involved in circadian rhythms is Clock, which can bind to the IL-23 receptor (IL-23R) promoter in γδ T cells. Mice harboring the loss-of-function mutation of the Clock gene developed reduced imiquimod-derived skin inflammation associated with a decreased of IL-23R expression in γδ T lymphocytes.[96]

Patients with psoriasis display increased homing of Vγ9Vδ2 T cells from the blood to the skin.[97] In addition, dermal Vγ9Vδ2 T cells found in patients with psoriasis secreted more IFN-γ, TNF-α, and IL-17A, inducing the recruitment of blood immune cells.[97] Importantly, psoriasis-targeted therapy reverted the decreased numbers of circulating Vγ9Vδ2 T cells, indicating its role in the disease.[97] However, the role of Vδ1 T-cell populations in patients with psoriasis has to be explored in depth. Further studies are also required to assess the potential role of the IL-23 responder subset CD26^{hi}CD94^{lo}Vδ2$^+$ T cells, which was recently identified.[66]

Atopic dermatitis

Atopic dermatitis (AD) is a prevalent chronic relapsing inflammatory skin disease that affects up to 20% of children worldwide. Both epidermal barrier dysfunction and immune dysregulation are associated with AD onset.[98] It is predominantly a T_H2 cell– and T_H22 cell–mediated inflammatory skin disease that also displays increased IL-17 expression, as in psoriasis.[99] Mice lacking SOX13, which are specifically deficient in Vγ4 but not in Vγ6 T cells, develop spontaneous AD.[4] Hence, dermal Vγ4 T-cell subset but not Vγ6 T-cell subset controls skin

commensal bacteria dysbiosis that alters the basal transcriptome of keratinocytes and induces IL-17– and IL-22–secreting αβ T-cell infiltration, thus preventing AD[100] (Fig 1). Importantly, both Vγ4 and Vγ6 T cells are affected in germ-free mice,[101] indicating the relevance of commensal pathogens in the generation and maintenance of these subsets. Mice deficient in FGF receptors 1 and 2 in keratinocytes display alterations in the epidermal barrier, leading to spontaneous AD. In these mice, the numbers of DETCs are increased and activated in the epidermis, but they do not participate actively in the development of AD caused by a barrier defect.[102]

Consistent with murine data, blood analysis demonstrates that patients with AD present with a lower proportion of γδ T lymphocytes than in healthy individuals.[103] Both circulating natural killer cell and γδ T-lymphocyte populations are decreased in patients with AD on account of apoptosis induced by monocytes, and they showed reduced secretion of TNF-α and IFN-γ cytokines but not IL-4.[104] However, children with AD display a significant increase in the frequency of Vγ9Vδ2 lymphocytes, highlighting the positive correlation between their expansion and severity of the disease. This increment may be due to the typical presence of *Staphylococcus aureus* in the skin of patients with AD.[105] The specific role and function of human skin–resident Vδ1 T cells in AD requires additional investigation.

Contact dermatitis

Contact dermatitis (CD) is the most frequent occupational skin disease (prevalence 95%); it can be divided into 2 types: (1) irritant contact dermatitis (ICD), which is caused by chemical or metal ions, and (2) allergic contact dermatitis (ACD), which is caused by the contact with allergens.[106]

The putative role of γδ T cells in contact hypersensitivity (CHS) model is controversial. Early studies showed that TCRδ$^{-/-}$ mice display an increased CHS reaction owing to γδ T-cell–mediated regulation of development of specific CD8 effector T lymphocytes and their cytotoxic activity.[107] On the other hand, some authors have observed that TCRδ$^{-/-}$ mice display less inflammation in a CHS model.[20,23] Differences observed regarding the role of γδ T cells in CHS models can reflect nonidentical experimental settings, but they can also be due to the differential role exerted by each subset of mouse skin γδ T cells.

During CHS, DETCs can migrate from the skin to draining lymph nodes at a very low rate, which is affected by deletion of occludin protein.[108] The mechanism for occludin-mediated regulation of DETC motility is not completely understood,[108] but occludin-deficient DETCs have not shown the morphologic changes required for migration. Hapten-activated keratinocytes can secrete IL-1β, which in combination with TCR activation, can promote DETC release of IL-17 and IFN-γ, thus promoting skin inflammation.[20] IL-1β signaling by DETCs also controls their activation and migration to the lymph node after skin sensitization.[20] DETC recognition of NKG2D ligands expressed by keratinocytes is required for allergen-induced activation of DETC and IL-17 secretion induced by IL-1β.[109] However, other studies have indicated a potential anti-inflammatory role of DETCs in CHS models. TCRδ$^{-/-}$ mice in the FVB background are more susceptible to development of ACD and ICD reaction than are TCRδ$^{-/-}$ mice in the C57BL6 background.[110] Adoptive transfer assays have demonstrated that the spontaneous dermatitis induced in TCRδ$^{-/-}$/FVB mice can be avoided by the transfer of selected Vγ5 T cells.[110] In addition, DETCs may inhibit ICD, reversing the increased croton oil reaction observed in mice deficient in ubiquitous homodimeric flavoprotein NAD(P)H:quinone oxidoreductase 1 (Nqo1).[111,112]

Although DETCs are clearly involved in the CHS response, dermal γδ T-cell subsets are the main source of IL-17 in the skin after hapten exposure.[23] Both the fractions Vγ4 T cells and Vγ6 T cells are rapidly increased after hapten application. Depletion of Vγ4 T cells clearly reduces the CHS response.[23] Thus, dermal γδ17 T cells play a relevant proinflammatory role in CHS response because they can promote the recruitment of neutrophils to hapten-treated skin in an IL-17–dependent manner.[23] The relative contribution of dermal Vγ6 versus Vγ4 T cells to the IL-17 secretion in CHS models has not been evaluated in depth.

In contrast to the number of mouse investigations, there are few studies in humans related to γδ T cells and ACD. γδ T cells isolated from ACD biopsy specimens from patients sensitized with corticosteroids secrete IL-4.[113] In patients with ACD, an early and sustained increment in the number of both Vδ1 and Vγ9Vδ2 T cells in the dermis and epidermis is observed after the contact with an allergen. These results indicate that although γδ T cells may not be participating in establishment of the disease, they may play relevant roles in the early reaction against allergens.[114] In particular, allergic and irritant reactions to gold chloride, mercuric chloride, and nickel in humans increase the frequency of Vγ9Vδ2 T cells.[115,116]

γδ T-CELL–MEDIATED DEFENSE AGAINST SKIN INFECTION

Cutaneous bacterial infection

S aureus is a gram-positive extracellular bacteria that causes frequent skin infections in humans.[117] During cutaneous challenge with *S aureus* in mice, dermal-resident γδ T cells are the major cellular source of IL-17 and IL-22, which are essential to host defense by promoting neutrophil recruitment and bacterial clearance.[118,119] Hence, TCRδ$^{-/-}$ mice showed larger lesions after infection, whereas TCRβ$^{-/-}$ mice had lesions similar to controls.[120] The release of IL-1β during intradermal infection with *S aureus* in mice promoted IL-17A/F responses by dermal γδ T cells, which are required for effective neutrophil recruitment and bacterial clearance.[121,122] However, epicutaneous *S aureus* exposure drove skin inflammation that was independent of IL-1β signaling, and mediated by bacterial PSMα and IL-36R/MyD88-induced production of IL-17 by γδ T cells and T_H17 cells.[123] Recently, it has been demonstrated that a population of Vγ6Vδ4 T cells secreting IL-17A, IL-22, TNF-α, and IFN-γ is clonally expanded in the lymph nodes and recruited to the skin to efficiently control *S aureus* infection in mice.[5] Whether these expanded cells originate from bone marrow precursors has not been specified. However, it is interesting to note that this clonal expansion to skin infection also involves the Vδ4 chain, as in the inflammatory responses after administration of imiquimod or CFA, highlighting the use of this Vδ chain as a potential marker of inducible and extrathymic γδ T cells.

During the initial phase of human infection with low bacterial inoculates of *S aureus*, Vγ9Vδ2 T cells are activated by endogenous phosphoantigens, and they readily respond to bacterial infection[124] (Fig 2). A seminal study using a chimeric severe combined immunodeficiency mouse model showed that

human Vγ9Vδ2 T cells mediate resistance to extracellular gram-positive (*S aureus*) and gram-negative (*Escherichia coli* and *M morganii*) bacteria.[125] Intravenous treatment of infected, reconstituted chimeric severe combined immunodeficiency mice with a specific aminobisphosphonate antigen for human Vγ9Vδ2 T cells markedly increased the *in vivo* antibacterial response.[125] Recently, it has been shown that *S aureus*–infected dendritic cells activate human Vδ2 T cells and induce IFN-γ secretion by a mechanism involving direct cell-cell contact and IL-12 secretion.[126] Also, the T_H1 cell response induced by *S aureus*–infected dendritic cells was increased when Vδ2 T cells were added to the culture.[126] Thus, circulating human Vδ2 T cells not only directly target bacterial load but can also be important for proper adaptive immune response.

During infection of the skin by *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), mouse dermal γδ T cells are also the predominant source of IL-17, and their absence is associated with decreased neutrophil recruitment to the skin.[13] Moreover, the absence of γδ T cells prevents the expansion in skin-draining lymph nodes of $CD4^+$ T cells specific for an immunodominant *Mycobacterium tuberculosis* epitope.[13] Use of Kaede transgenic mice, which allow *in vivo* tracing of peripheral versus circulating cells,[127] revealed that dermal Vγ4 T cells migrate to draining lymph nodes and modulate CD8 T-cell activity through TNF-α production in a model of BCG dermal infection.[26] These observations indicate that the main function of dermal γδ T cells in this context is to act as a bridge between innate and adaptive immune responses against bacterial skin infections.

Vaccine against *M bovis* BCG is administered to more than 120 million infants each year worldwide. Mycobacterial antigens dramatically expand the number of Vγ9Vδ2 T cells *in vitro*, which is significantly enhanced in BCG responders compared with subjects who were not sensitized, indicating that BCG vaccination induced a memory-like phenotype in Vγ9Vδ2 T cells.[128] Although most of studies investigating the immune response to BCG have been focused on αβ T cell–mediated adaptive response, Vγ9Vδ2 T cells are the key circulating population producing IFN-γ in response to BCG immunization in infants and children.[129]

Cutaneous fungal infection

Candida albicans is a dimorphic commensal fungus that naturally inhabits the skin, genital mucosa, and/or intestinal mucosa in up to 70% of healthy individuals.[130] Dermal γδ T cells are the main producers of innate IL-17 response to experimental infection with *C albicans*.[131] Hence, c-Maf–deficient mice, which have a normal DETC subset but both Vγ4 and Vγ6 T cells are completely absent, are more susceptible to *C albicans* infection.[43] The numbers of human Vδ1 T cells are expanded in patients with HIV infection and candidiasis, and they produce IL-17 in response to IL-23.[132]

Commensal fungi of the mammalian skin, such as those of the genus *Malassezia*,[133] are associated with AD and other common inflammatory skin disorders.[134] Epicutaneous application of *Malassezia* demonstrated that the IL-23/IL-17 axis and γδ T cells play a central role in stimulating and/or exacerbating the inflammatory response.[135] Recently, it has been demonstrated that cutaneous colonization with commensal skin fungi such as *C albicans* and *Malassezia furfur* is associated with the induction of highly polarized type 17 skin immune responses that potentiate the imiquimod-induced inflammation.[136]

Cutaneous virus infection

Vaccinia virus is a member of the poxvirus family, which has been used successfully as a vaccine to eradicate human smallpox. The population of dermal γδ T cells is increased 10-fold in a murine model of cutaneous vaccinia virus infection.[137] $CD27^+$ and $CD27^-$ γδ T cells were specifically recruited from the circulation to the site of infection, but they do not become resident populations in the tissue. Recruited $CD27^+$ γδ T cells secreting granzyme B were required to control viral replication and inflammatory response. Conversely, dermal γδ17 T cells locally enhanced the inflammatory response.[137] Vaccinia vaccination induced robust memory effector CD4, CD8, and circulating IFN-γ–producing γδ T cells in humans, all of which are considered relevant for protection against smallpox[138] (Fig 2).

Mice depleted of or deficient in γδ T cells showed severe herpes simplex virus 1 (HSV-1)–induced epithelial lesions.[139] However, the role of different populations of skin-resident γδ T cells secreting IL-17 has not been analyzed in cutaneous lesions of HSV-1, as has been done in corneal infection, in which $CCR6^+IL\text{-}1R^+IL\text{-}23R^+$ γδ T cells mediate protection.[140] Cutaneous HSV-1 infection is observed first in keratinocytes and DETCs that transport viral antigens from the skin to the draining lymph node (ie, before Langerhans cell infection and migration).[141] However, the relevance of these process is not completely understood. HSV-1–seropositive individuals contain elevated numbers of γδ T cells in their peripheral blood, indicating specific recognition and expansion.[142]

ROLE OF γδ T CELLS IN MALIGNANT CUTANEOUS DISEASES

Skin cancer is one of the most common cancers worldwide, and its incidence has increased continuously in the past few decades.[143] γδ T cells exert antitumor activity through the direct lysis of cancer cells by the perforin-granzyme pathway and by the release of IFN-γ and TNF-α, which enhance antitumor immunity and inhibit angiogenesis. Moreover, γδ T cells but not αβ T cells express CD16, the low affinity Fc receptor for IgG (FcγRIII). Thus, γδ T cells can directly kill cancer cells via antibody-dependent cellular cytotoxicity and through the ligands TRAIL and FasL.[6]

Mice lacking γδ T cells are more susceptible to development of cutaneous tumors, demonstrating the pivotal role of this cell type in cancer immunosurveillance.[144] DETCs are the unique population of skin γδ T cells that express NKG2D, which in addition to the TCR, can mediate the direct recognition and lysis of malignant cells in chemical- or ultraviolet-induced cutaneous malignances, as well as in transplanted tumor cell line models, such as melanoma.[145-147] The secretion of IFN-γ by DETCs is also important for the antitumor response because it promotes the recruitment and activation of other cytotoxic lymphocytes such as natural killer and CD8 T cells.[148] However, γδ T cells can also exert protumor activity, which is mainly related to their capacity of IL-17 secretion. Indeed, γδ T cells are the primary source of IL-17 within the tumor microenvironment, where they promote tumor growth by increasing angiogenesis[149] and recruitment of myeloid-derived suppressor cells.[150] A population of

mouse IL-17A–secreting Vδ1 T cells, which express CD30 and use both Vγ1 and Vγ6 chains, has been relevant to orchestrate an inflammatory microenvironment leading to cancer progression and metastasis.[151]

Human γδ T cells, which express either Vγ9/Vδ2 or Vδ1 TCRs, have been found within tumor-infiltrating lymphocytes in many cancers, including skin malignant lesions.[152,153] However, the positive or negative correlation of γδ T-cell infiltration with tumor growth, or their prognostic value, remains unclear (Fig 2). γδ T-cell–based immunotherapies include *in vitro* or *in vivo* expansion of IFN-γ-secreting Vγ9Vδ2 T cells (reviewed in Lo Presti et al[154]), which has been explored from the standpoint of use against various tumors of hematologic and epithelial origin.[155] Several clinical trials have shown that the use of Vδ2 T cells for immunotherapy is feasible and safe, although they have limitations with regard to effectiveness.[156] The possible usefulness of Vδ1 cells in immunotherapy has been less studied, although they could be programmed to treat some malignant diseases.[6,59,157]

γδ T cells recognize antigens not presented by MHC. However, human γδ T cells recognizing melanoma-associated antigens (MART1 and gp100) in an MHC-restricted fashion were recently generated *in vitro*.[158] This study revealed that classical MHC-restricted human γδ T cells can be found in the periphery and have the potential to be used to develop new TCR-based strategies against cancer.[158]

γδ T cells represent the major infiltrating lymphocyte population in melanoma, and both Vδ1 and Vδ2 cells are detected.[153] Polyclonal γδ T-cell lines obtained from melanoma samples produced IFN-γ and TNF-α and were capable of killing melanoma cell lines in vitro. Moreover, increased tumor infiltration of the human Vδ2 subset in the tumor cells is positively correlated with early-stage melanoma and absence of metastasis.[153] In patients with squamous cell carcinoma, tumor-infiltrating γδ T cells secrete IL-17 or IFN-γ depending on the tumor stage, whereas circulating γδ T cells of patients with squamous cell carcinoma preferentially produce IFN-γ.[159] These findings suggest the possibility that γδ T cells in squamous cell carcinoma are recruited from the periphery and are reprogrammed by the tumor microenvironment. In this regard, tumor hypoxia can induce differentiation of IL-17–secreting γδ T cells, and it reduces calcium efflux and expression of the degranulation marker CD107a, thus decreasing the antitumor cytotoxicity activity of γδ T cells.[160] This suggests that mechanisms involved in hypoxia-induced immunosuppression target differentiation of infiltrating γδ T cells.[160] Development of cancer has been linked to chronic inflammation, particularly via IL-23 and IL-17 signaling pathways.[161,162] A higher expression of IL-17 and IL-23 was found in melanoma biopsy specimens than in benign nevi, suggesting their possible involvement in the evolution of cutaneous melanomas and the development of invasive capability.[163] Hence, the described dual effect of γδ T cells in cancer diseases is mainly due to their ability to secrete either IFN-γ or IL-17, which should be targeted by the future immunotherapy strategies.

CONCLUDING REMARKS AND PERSPECTIVES

Since their discovery, γδ T cells have been the focus of attention of immunologists, with a particular emphasis on skin diseases. Because of the increased complexity of γδ T-cell subsets in mouse skin, efforts have been made to identify which specific Vγ use is relevant for each skin pathology. However, data indicate that even γδ T cells bearing identical Vγ chains can be considered different subsets from the standpoints of their origin and localization. Further studies identifying specific markers that distinguish bone marrow–derived Vγ4 cells from those that require fetal cells for reconstitution are mandatory. Similarly, deep analysis of skin human γδ T cells detected in homeostasis and in different diseases will undoubtedly improve novel therapeutic strategies.

Although γδ T cells are considered to be innate cells, evidence of clonal expansion and resident memory behavior has already been documented in skin diseases, including psoriasis and infection. Interestingly, TCR-mediated recognition of specific antigens has been described at least for melanoma cancer cells. Conceivably, γδ T can become adaptive to increase the skin protection against cancer and to establish long-lived memory responses to pathogens. An outstanding question is whether TCR-mediated specific recognition of antigens, in addition to stimulation with proinflammatory cytokines, is required for a γδ T-cell response during skin inflammation. To further understand the mechanism mediating γδ T-cell functions, novel genetic strategies specifically targeting different subsets of skin γδ T cells, as well as the molecules involved in antigen recognition, need to be explored in murine models of psoriasis, dermatitis, cancer, and infection. Finally, although the role of γδ T cells as a bridge between innate and adaptive immune cells has been explored, the possible interaction between γδ T cells and different subtypes of resident innate cells in the skin, including dendritic cells and macrophages, has not been explored, either in homeostasis or in inflammation. Knowledge of the molecular mechanisms that define γδ T cells' function in skin homeostasis and skin diseases will identify novel strategies to improve the resolution of inflammation.

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